Constanza Estefany Fernández González

EFEITO DE DENTIFRÍCIO FLUORETADO E APLICAÇÃO PROFISSIONAL DE FLUORETO NO CONTROLE DE CÁRIE DE ESMALTE E DE DENTINA RADICULAR

EFFECT OF FLUORIDATED TOOTHPASTE AND PROFESSIONAL FLUORIDE APPLICATION ON CARIES CONTROL IN ENAMEL AND ROOT DENTINE

PIRACICABA - SP

2015

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

Constanza Estefany Fernández González

EFEITO DE DENTIFRÍCIO FLUORETADO E APLICAÇÃO PROFISSIONAL DE FLUORETO NO CONTROLE DE CÁRIE DE ESMALTE E DE DENTINA RADICULAR

EFFECT OF FLUORIDATED TOOTHPASTE AND PROFESSIONAL FLUORIDE APPLICATION ON CARIES CONTROL IN ENAMEL AND ROOT DENTINE

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Odontologia, na Área de Cariologia.

Thesis presents to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dentistry, in Cariology area.

Orientador: Prof. Dr. Jaime Aparecido Cury

Este exemplar corresponde à versão final da tese
defendida pela aluna Constanza Estefany Fernández González
e orientada pelo Prof. Dr. Jaime Aparecido Cury.

_____

Assinatura do Orientador

PIRACICABA - SP

2015

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

F391e

Fernández, Constanza Estefany, 1985-
Efeito de dentifrício fluoretado e aplicação profissional de fluoreto no controle de cárie de esmalte e de dentina radicular / Constanza Estefany Fernández González. – Piracicaba, SP : [s.n.], 2015.

Orientador: Jaime Aparecido Cury.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Desmineralização. 2. Remineralização dentária. 3. Fluoreto de fosfato acidulado. 4. Cremes dentais. I. Cury, Jaime Aparecido,1947-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Effect of fluoridated toothpaste and professional fluoride application on caries control in enamel and root dentine
Palavras-chave em inglês:
Demineralization
Tooth remineralization
Acidulated phosphate fluoride
Toothpastes
Área de concentração: Cariologia
Titulação: Doutora em Odontologia
Banca examinadora:
Jaime Aparecido Cury [Orientador]
Rodrigo Andrés Giacaman Sarah
Carolina Patrícia Aires
Maria da Luz Rosario de Sousa
Cassiano Kuchenbecker Rosing
Data de defesa: 10-02-2015
Programa de Pós-Graduação: Odontologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 10 de Fevereiro de 2015, considerou a candidata CONSTANZA ESTEFANY FERNANDEZ GONZALEZ aprovada.

Prof. Dr. JAIME APARECIDO CURY

Prof. Dr. RODRIGO ANDRÉS GIACAMAN SARAH

Profa. Dra. CAROLINA PATRICIA AIRES

Profa. Dra. MARIA DA LUZ ROSARIO DE SOUSA

Prof. Dr. CASSIANO KUCHENBECKER ROSING

RESUMO

Dentifrício fluoretado (DF) de concentração convencional (1000-1500-ppm) é eficiente no controle da cárie de esmalte, entretanto seu efeito na cárie radicular pode não ser o mesmo devido à maior susceptibilidade da dentina à cárie. Essa diferença poderia ser compensada pelo uso diário de DF de maior concentração (DF-5000-ppm) ou da combinação da aplicação profissional de fluoreto (APF) com uso diário de DF-1100-ppm. Entretanto, além dessa comparação nunca ter sido pesquisada, a diferença do efeito entre esmalte e dentina tem sido pouco estudada num único experimento. Em acréscimo, o efeito de DF-5000-ppm na dentina radicular se limita a estudos do efeito da remineralização. O **Capítulo 1** dessa tese descreve a validação do modelo de biofilme de *Streptococcus mutans* (SM) para estudar o efeito dose resposta de DF na redução da desmineralização (des-) do esmalte e dentina radicular. No **Capítulo 2**, foi avaliado *in situ* o efeito dose-resposta de DF e o efeito de DF-5000-ppm ou a combinação de APF com DF-1100-ppm na redução da des- e ativação da remineralização do esmalte e dentina radicular. No capítulo 1, biofilmes de SM foram crescidos sobre blocos de esmalte e dentina radicular, os quais foram expostos à sacarose 8x/dia e 2x/dia às soluções fluoretadas contendo 0, 150, 450 e 1350-ppm. Ao final do experimento, a concentração de F foi determinada nos biofilmes e nos blocos a dureza de superfície (DS) e F-solúvel em ácido (FAp). No capítulo 2, num desenho cruzado, duplo-cego de 4 fases de 14 dias cada, 18 sujeitos utilizaram dispositivo palatino contendo blocos hígidos e cariados de esmalte e de dentina radicular. Biofilme foi permitido ser acumulado sobre os blocos, os quais foram expostos à sacarose 8x/dia ou 3x/dia, nos blocos hígidos ou cariados, respectivamente. Os tratamentos foram: 1) Dentifrício-placebo (DP); 2) DF-1100; 3) DF-5000; 4) único pré-tratamento dos blocos com APF (gel acidulado, 12300-ppm) combinado com DF-1100 (APF+DF-1100). Dentifrícios foram usados 2x/dia. No fluido e porção sólida do biofilme foram determinadas as concentrações de F. Nos blocos a porcentagem de perda (%PDS) ou recuperação da DS (%RDS), nos substratos hígidos e cariados respectivamente, como também as áreas de lesão de cárie (ΔS). F-solúvel em álcali e ácido também foram determinadas nos blocos. Os resultados *in vitro* mostraram que o modelo apresentou efeito dose-resposta, havendo em função da concentração de F um aumento de F no biofilme e um aumento da concentração de FAp e decréscimo da %PDS nos blocos de esmalte-dentina. O efeito do F na redução da desmineralização no estudo *in vitro* foi menor na dentina do que o observado no esmalte. Os dados

in situ mostraram efeito dose-resposta para a concentração de F nos dentifrícios (0;1100;5000) para a maioria das variáveis analisadas, à exceção do ΔS para dentina-cariada. Na comparação dos tratamentos, APF+DF-1100 foi mais eficaz que DF-5000 reduzindo desmineralização e ativando a remineralização da dentina radicular. Os resultados *in vitro e in situ* permitiram concluir que: a) Um modelo de biofilme foi validado para estimar o efeito dose-resposta de DF na redução da desmineralização do esmalte e dentina radicular; b) O efeito de DF no controle de cárie de esmalte ou dentina é concentração dependente, sendo a combinação com APF mais relevante para dentina-radicular que para esmalte.

Palavras-chaves: Desmineralização. Remineralização dentária. Biofilme dentário. Flúorfosfato acidulado. Creme dental.

ABSTRACT

Fluoride dentifrice (FD) of conventional concentration (1,000-5,000 ppm) is efficient for enamel caries control; however, it is possible that their effect might not be the same for root-caries because dentine is more caries susceptible. This difference could be compensated by the daily use of high concentration FD (5,000 ppm-FD) or by the combination of professional fluoride application with daily use of 1,100 ppm-FD. Besides, this comparison has never been researched; the difference of effect between enamel and dentine has been scarcely evaluated in the same experiment. In addition, the effect of 5,000-FD has been focused essentially on root-caries remineralization. **Chapter 1** of this thesis describes the validation of a *Streptococcus mutans* (SM) biofilm model to study the dose-response effect of FD in the reduction of demineralization (de-) on enamel and root-dentine**.** In **Chapter 2**, it was evaluated *in situ* the FD dose-response effect and the effect of FD-5000 ppm or the combination of acidulated phosphate fluoride (APF) application with DF-1100 ppm in the reduction of de- and activation of remineralization on enamel and root-dentine. In chapter 1, SM biofilms were grown on enamel and root-dentine slabs, and exposed 8x/day to sucrose and 2x/day to fluoridated solutions containing 0, 150, 450 and 13,50-ppm. At the end of the experiment, F-concentration in the biofilms was determined and surface hardness (SH) and acid-soluble-F (FAp) were assessed in the slabs. In chapter 2, in a cross-over and blind design of 4 phases of 14 days each, 18 subjects wore a palatine appliance containing sound and carious slabs of enamel and root-dentine. Biofilm was allowed to accumulate on the slabs and while the sound slabs were exposed to sucrose 8x/day the carious were exposed 3x/day. The treatment groups were: 1) F-placebo dentifrice (PD); 2)1,100-FD; 3) 5,000-FD; 4) slabs pre-treated with APF (F-gel, 12,300 ppm-F) combined with daily use of 1100-FD (APF+1,100-FD). Dentifrices were used 2x/day. The F concentration was determined in fluid and solid portions of the biofilm. In slabs, the percentage of SH loss (%SHL) or recovery (%RSH) was estimated in sound and carious slabs, respectively, as well as the caries lesion area (ΔS). Acid-soluble-F and alkali-soluble-F concentrations were also determined in the slabs. The *in vitro* results showed that the model presented a dose-response effect, with an increment of F concentration in the biofilm and an increment of FAp in function of F concentration of the treatments. Also, %SHL showed a reduction in enamel and dentine slabs according to the treatments. F effect on the reduction of demineralization was lower on dentine than that observed on enamel. The *in situ* data showed a

dose-response effect to F concentration in the dentifrices (0;1,100;5,000) for most response variables, with the exception of ΔS for carious-dentine. In the treatments comparison, APF+1100-FD was statistically more effective than 5000-FD at reducing demineralization and activating remineralization in root-dentine. The *in vitro* and *in situ* results gave support to the conclusions: a) A biofilm model was validated to estimate the dose-response effect of FD in demineralization reduction either on enamel and root dentine; b) The FD effect in enamel and dentine caries control is concentration-dependent, but the combination with APF was more relevant to root-dentine than to enamel.

Keywords: Demineralization. Dental remineralization. Dental biofilm. Acidulated phosphate fluoride. Toothpaste.

SUMÁRIO

DEDICATÓRIA

A Deus por me dar a vida e tantas oportunidades.

À minha família e amigos que na distância me deram seu incentivo,

carinho e força para essa conquista.

AGRADECIMENTOS

Ao Magnífico Reitor da Universidade Estadual de Campinas, Prof. Dr. **José Tadeu Jorge**.

À Faculdade de Odontologia de Piracicaba, na pessoa do seu diretor, Prof. Dr. **Guilherme Elias Pessanha Henriques**

À Profa. Dra. **Cínthia Pereira Machado Tabchoury**, coordenadora dos Cursos de Pós-graduação da Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas.

À Profa. Dra. **Juliana Trindade Clemente Napimoga**, coordenadora do Curso de Pós-graduação em Odontologia, Faculdade de Odontologia de Piracicaba.

Ao Prof. Dr. **Jaime Aparecido Cury**, meu mestre e orientador, por todos os ensinamentos e o tempo dedicado na minha formação, pela confiança depositada, pelas oportunidades, pelos conselhos e a paciência. Exemplo de ética, trabalho e paixão pela pesquisa e docência.

À Profa. Dra. **Livia Maria Andaló Tenuta**, meu exemplo de Professora, minha mestre, toda a minha gratidão por todo o tempo dedicado na minha formação e pelo grande trabalho e ajuda nesta tese. Agradeço a atenção, disposição, apoio e conselhos para me fazer uma melhor estudante e profissional.

À Profa. Dra. **Altair Antonina Del Bel Cury**, pelos ensinamentos, pelas discussões científicas e o tempo dedicado à minha pesquisa.

À Profa. Dra. **Cínthia Pereira Machado Tabchoury**, pelos ensinamentos, pelas discussões científicas tudo o que ela acrescentou durante toda a minha formação durante a pós-graduação. Exemplo de responsabilidade, dedicação e excelente disposição.

Ao Prof. Dr. **Rodrigo Giacaman Sarah** meu motivador e impulsionador neste desafio, exemplo de paixão pela pesquisa e docência. Agradeço pelas oportunidades, pelo apoio e pela grande confiança depositada em mim.

Aos **Profs. Drs. Pedro Luiz Rosalen, Cínthia Pereira Machado Tabchoury e Antônio Pedro Ricomini Filho** pelas considerações feitas durante o exame de qualificação.

Aos Profs. Drs. **Carlos González Cabezas, Margherita Fontana, Alexander Rickard e Susan Flannagan** pela acolhida e pelos ensinamentos durante o estágio realizado na Universidade de Michigan, EUA durante o ano 2014, como parte do programa de Doutorado em Odontologia da FOP. UNICAMP.

Aos Srs. **Waldomiro Viera Filho** e **Alfredo José da Silva**, técnicos do Laboratório de Bioquímica, pela ajuda, pela disposição e pela grata convivência durante todos estes anos.

Aos **colegas** do Programa de Pós-graduação em Odontologia da Faculdade de Odontologia de Piracicaba. Ao **Junior Ribeiro** pela grande amizade, alegria e ajuda de sempre. À **Juliana Botelho** pela grande ajuda e disposição, ao **Diego Nóbrega** pelo companheirismo, amizade, o trabalho em equipe e apoio. Aos demais colegas do programa pelo companheirismo e amizade, **Amanda, Bárbara, Dayse, Karla, Marília, Pablo e Tarcísio.**

Aos **colegas** de outras áreas da Pós-graduação e graduação da Faculdade de Odontologia de Piracicaba, **Airton, Alicia, Aline, Amanda, Cassiano, Cínthia, Dayse, Diego, Eloá, Gian Carlo, Guilherme, Heloisa, Indira, Juliana, Larissa, Marisol, Paulo Campos, Priscila, Rayane, Renan, Renato, Renata, Talhes, Yuri, Jéssica.** Especialmente aqueles que foram meus voluntários na pesquisa, minha eterna gratidão.

Ás minhas amigas **Alicia Rumayor, Marisol Martinez, Camila Concha, Marisol Miranda**, que foram minha família nesta cidade**.** À **Alicia** minha meia-irmã, pela sincera amizade, pelo apoio, preocupação e todo o tempo que convivemos.

A todos que direta ou indiretamente contribuíram para a realização deste trabalho e participaram da minha vida durante a pós-graduação.

EPÍGRAFE

"Live as if you were to die tomorrow. Learn as if you were to live forever."

Mahatma Gandhi

"Faith is taking the first step even when you don't see the whole staircase."

Martin Luther King Jr

"***Education is the most powerful weapon which you can use to change the world."***

Nelson Mandela

LISTA DE FIGURAS E TABELAS

CAPÍTULO 1

CAPÍTULO 2

LISTA DE ABREVIATURAS E SIGLAS

Português

F – fluoreto

DF – dentifrício fluoretado

APF – aplicação profissional de fluoreto

FFA – flúor fosfato acidulado

des – desmineralização

re – remineralização

%PDS – porcentagem de perda de dureza de superfície

%RDS – porcentagem de recuperação de dureza de superfície

ΔS – área de lesão de cárie

ppm –partes por milhão

Inglês

F – fluoride

FD – fluoride dentifrice

APF – acidulated phosphate fluoride

de – demineralization

re – remineralization

%SHL – percentage of surface hardness loss

%SHR – percentage of surface hardness recovery

ΔS – area of caries lesion

ppm –parts per million

INTRODUÇÃO

Com o concomitante declínio da cárie coronária (Bratthall et al., 1996) e o aumento da longevidade das populações (Petersen e Yamamoto, 2005), o desafio no futuro será de simultaneamente manter a cárie de esmalte sob controle com a prevenção de cárie na dentina radicular exposta (Curzon e Preston, 2004). Considerando a conservação de dentes naturais por mais tempo e a consequente possibilidade de superfícies radiculares expostas, a cárie radicular será um problema que aumentará no futuro (Jones, 1995; Tan et al., 2010; Walls e Meurman, 2012), porque em acréscimo, a dentina é mais suscetível à cárie que o esmalte (Nyvad e Fejerskov, 1982; Wefel, 1994). Entretanto, a pesquisa por agentes preventivos tem se centrado na cárie de coroa (esmalte), a qual afeta principalmente crianças e jovens, e não na radicular a qual afeta não somente idosos como também adultos de meia idade (Tan et al., 2010; Rodrigues et al., 2011).

O controle de toda doença deve estar direcionado ao controle dos fatores etiológicos envolvidos, assim na cárie, tanto do esmalte como da dentina, os principais são a remoção/desorganização do biofilme e aconselhamento dietético. No entanto, o sucesso destas estratégias tem sido limitado, sendo a cárie uma doença não erradicável, mas controlável. O uso de medidas preventivas e terapêuticas não invasivas é importante especialmente em idosos (McGrath et al., 2009), para reduzir a rápida progressão de lesões de cárie radicular, as quais atingindo estágios mais avançados são de difícil tratamento restaurador (Stamm et al., 1990), tem maior risco de recorrência (Fure et al., 1998) ou podem requerer frequente troca de restaurações (Curzon e Preston, 2004). Além disso, grande parte da população idosa não tem percepção da necessidade de tratamentos preventivos e só procura o atendimento odontológico para tratamento curativo (Du et al., 2009). Tudo isso justifica a necessidade de medidas para o controle precoce da cárie radicular.

A utilização do fluoreto é considerada o principal fator determinante positivo e o método mais indicado para controlar cárie (Cury e Tenuta, 2008). Assim, embora o fluoreto não seja capaz de interferir nos fatores responsáveis pela doença, ele é extremamente eficiente em reduzir sua progressão. Da mesma forma que o fluoreto é importante para o esmalte, o efeito dele na redução da progressão e na reversão de lesões de dentina radicular, faz com que sua utilização seja uma das

principais estratégias para o controle não invasivo neste substrato (Griffin et al., 2007; Heijnsbroek et al., 2007; Petersson, 2013; Wierichs e Meyer-Lueckel, 2014). Para isto, é importante que o fluoreto esteja presente constantemente no local certo (fluido do biofilme, saliva) e no momento certo (quando da exposição à sacarose), na sua forma livre e solúvel para interferir diretamente com os processos de des-remineralização do esmalte e dentina. Ele atua, diminuindo a quantidade de minerais perdidos quando ocorre o fenômeno da desmineralização e aumentando a resposta na remineralização, sendo efetivo mesmo em baixas concentrações (Cury e Tenuta, 2008, 2009; Tenuta e Cury, 2010).

Considerando que os estágios iniciais de desenvolvimento da cárie radicular são essencialmente similares à cárie de esmalte (Wefel, 1994), o F age por um mesmo mecanismo de ação (Featherstone, 1994) e ambos os substratos são beneficiados pela ação do fluoreto. Entretanto, o conhecimento sobre o efeito anticárie do fluoreto na dentina é menor que no esmalte (ten Cate e Featherstone, 1991; Featherstone, 1994; ten Cate et al., 1998). Como dito, a dentina é mais susceptível à cárie que o esmalte, assim as lesões de cárie progridem mais rapidamente nela que no esmalte (Ogaard et al., 1988). Isto é devido a alguns fatores, como o maior pH crítico necessário para a desmineralização (Hoppenbrouwers et al., 1986; Wefel, 1994), a maior porcentagem de matriz orgânica (Kawasaki e Featherstone, 1997), a maior permeabilidade (ten Cate et al., 1998) e pela presença de cristais minerais menores com maior concentração de carbonato (Nyvad e Fejerskov, 1982) que o esmalte. Além do mais, como o pH crítico para a desmineralização da dentina é superior ao do esmalte, sua dissolução se inicia antes e se prolonga por maior período de tempo (Curzon e Preston, 2004; Cury et al., 2011). Neste contexto de diferenças entre estes substratos, estudos sugerem que o efeito do F não seria na dentina da mesma magnitude que a encontrada para o esmalte (Featherstone et al., 1983; Herkstroter et al., 1991; ten Cate, 1999), porém a literatura é carente de estudos feitos com dentina isolada ou sua comparação direta com o esmalte, quando eles são submetidos à cárie no mesmo experimento.

Assim, foi mostrado *in vitro* que é necessária uma maior quantidade de fluoreto (F) para a inibição da desmineralização (Herkstroter et al., 1991) ou ativação da remineralização (Featherstone et al., 1983) da dentina que a do esmalte. Também há publicações sugerindo que maiores concentrações de F (ten Cate et al., 1995; Baysan et al., 2001; Mukai et al., 2001;

Heijnsbroek et al., 2007; Ekstrand et al., 2013; Srinivasan et al., 2014) ou maior frequência de uso (Laheij et al., 2010) seriam necessárias para controlar cárie de dentina radicular. No caso do esmalte, o uso de dentifrício fluoretado (DF) tem sido proclamado o fator mais importante na diminuição da prevalência da cárie ocorrida em países desenvolvidos (Bratthall et al., 1996) e em desenvolvimento (Cury et al., 2004), sendo a sua eficácia anticárie baseada em evidência para controle de cárie em crianças e adolescentes (Marinho et al., 2003; Walsh et al., 2010). Ele tem sido considerado o meio mais racional de uso de F, pois ao mesmo tempo que o biofilme é desorganizado pela escova, ocorre disponibilização de fluoreto para o meio bucal (Kidd e Fejerskov, 2013; Cury e Tenuta, 2014). No entanto, para dentina os dados da literatura sugerem fortemente que para o DF ser eficaz na redução de desmineralização ou ativação da remineralização, ele não pode ser usado da mesma maneira que para o esmalte, quer seja em termos de concentração ou combinando seu uso com outros meios de utilização de fluoretos. Entretanto, a maioria dos estudos realizados avaliou o efeito do fluoreto na reparação de lesões de cárie (ativação da remineralização) (Nyvad et al., 1997; Baysan et al., 2001; Mukai et al., 2001; Ekstrand et al., 2013; Srinivasan et al., 2014), mas não na iniciação e progressão das lesões (inibição da desmineralização).

A respeito da cárie coronária (de esmalte), embora esta tenha declinado (Bratthall et al., 1996), ela não tem sido totalmente controlada (Bagramian et al., 2009) especialmente em pacientes de alto risco. Assim, o uso de maiores concentrações de F, como DF de alta concentração (5000 ppm F) (Nordstrom e Birkhed, 2010; Sonesson et al., 2013) ou a associação de produtos (Zimmer et al., 2001) têm sido recomendados para populações de maior risco. Revisões sistemáticas da literatura e estudos experimentais *in situ* têm mostrado que para o esmalte, a combinação de outros meios de uso de F tópico (p.e. aplicação profissional), em acréscimo ao uso de dentifrício fluoretado, provoca um benefício adicional modesto (não significativo) de redução de cárie do que o efeito isolado do dentifrício (Marinho et al., 2004; Paes Leme et al., 2004). Entretanto, para controlar a cárie radicular há dados sugerindo que a combinação de dentifrício com aplicação profissional de fluoreto (APF) é mais eficaz que o efeito isolado desses meios (Nyvad et al., 1997; Vale et al., 2011).

Estas duas estratégias, seja o uso diário de DF de alta concentração (DF-5000 ppm) ou o uso de produtos de APF combinado com o uso diário de DF de concentração convencional (DF-1100 ppm), tem o objetivo de dar uma contribuição adicional de F quando o uso diário de DF-1100 ppm não é suficiente para reduzir a desmineralização ou ativar a remineralização. Durante a escovação com dentifrício fluoretado se produz um aumento momentâneo da concentração de F na boca (saliva e biofilme), e na medida que o tempo passa ocorre uma diluição do F remanescente na saliva pelo *clearance* salivar. No entanto, o F pode ficar retido no biofilme não removido ou em superfícies limpas pela escovação. Quando maiores concentração de F são utilizadas, além desse aumento de F na saliva, pode ocorrer a formação de produtos de reatividade nas superfícies dentárias na forma de mineral tipo fluoreto de cálcio ("CaF_2"; também chamado de F solúvel em álcali ou flúor fracamente ligado). Para DF de concentração convencional, a formação desses produtos não seria relevante (Tenuta e Cury, 2013), mas poderia ser para DF-5000 ppm F. No caso de produtos de aplicação profissional de fluoreto (APF) a formação destes reservatórios tem sido considerado o principal mecanismo de ação anticárie (Tenuta et al., 2008), o qual disponibiliza fluoreto para interferir com a des-remineralização de esmalte ou dentina. No entanto, o efeito de DF de alta concentração comparado com APF combinado com uso diário de DF de concentração convencional, não tem sido estudado.

Pelo exposto, e considerando que a dentina em relação ao esmalte é mais suscetível à cárie, que a recessão gengival expõe simultaneamente o esmalte e tecido radicular adjacente às mesmas condições ambientais de risco/benefício, e que o efeito comparativo entre DF-5000 ppm e APF associado a DF-1100 ppm não tem sido descrito, foram conduzidos dois estudos. Primeiramente foi avaliado *in vitro* o efeito do DF na redução da desmineralização (des-) no esmalte e dentina radicular através da validação do modelo de biofilme de *Streptococcus mutans* em termos de dose-resposta **(Capítulo 1).** Em seguida, foi avaliado *in situ* o efeito dose-resposta de DF (0; 1100; 5000 ppm F) e o efeito de DF-5000 ppm ou a combinação de APF com DF-1100 ppm na des- e remineralização do esmalte e dentina radicular **(Capítulo 2).** Os dados gerados nesta tese contribuem para o entendimento do efeito do fluoreto no controle da cárie de esmalte e de dentina radicular.

CAPÍTULO 1

Biofilm model to evaluate the effect of fluoride on enamel and on root dentine demineralization[1]

Fernández C.E., Tenuta L.M.A., Cury J.A.

Piracicaba Dental School, UNICAMP, Piracicaba, São Paulo, Brazil.

Short title: Cariogenic biofilm model to study fluoride effect

Corresponding author:
Prof . Jaime A. Cury
Piracicaba Dental School
Av. Limeira, 901 (CP 52)
13.414-903 Piracicaba SP
Brazil
Tel. +55 19 3412 5302/5303, Fax +55 19 3412 5218
e-mail jcury@unicamp.br

Declaration of interest

There are no conflicts of interest with respect to the authorship and/or publication of this article.

[1] *Artigo submetido para publicação no periódico Caries Research. Comprovante da submissão no anexo 2.*

Biofilm model to evaluate the effect of fluoride on enamel and on root dentine demineralization

ABSTRACT

Enamel and root dentine are concurrently at a risk of developing caries due to gingival recession and biofilm accumulation in dental cervical area, but there is no validated model to evaluate the simultaneous effect of fluoride on these dental substrates, reason of the present study. *Streptococcus mutans* UA159 biofilms were formed on saliva-coated bovine enamel and root dentine slabs mounted in the same well of culture plates. The biofilms were exposed 8×/day to 10% sucrose and treated 2×/day with fluoridated solutions containing 0, 150, 450, or 1,350 ppm F; thus, simulating the use of low to high fluoride concentration toothpastes. The pH values of the culture medium was monitored 2×/day as a biofilm acidogenicity indicator. After 96 h, biofilms were collected for fluoride concentration analysis. The percentage of surface hardness loss (%SHL) was calculated for slabs. The fluoride uptake by the enamel and dentine was also determined. The model showed a dose-response because the biofilm and fluoride uptake increased and %SHL decreased at increasing fluoride concentrations ($p < 0.05$). Fluoride in the biofilm formed on dentine and fluoride uptake by dentine were higher than those for enamel. However, with the same fluoride concentration treatment, the percentage of reduction of demineralization was lower for dentine than for enamel. In conclusion, the model was validated in terms of a dose-response effect of fluoride on enamel and root dentine. Furthermore, the findings support the clinical data, suggesting that higher fluoride concentrations are necessary to control caries of root dentine than of enamel.

Keywords: dentifrices, *Streptococcus mutans*, dental caries, toothpastes, fluoride

INTRODUCTION

The decrease of caries prevalence and the increase in life expectancy [Petersen and Yamamoto, 2005] allow more natural teeth to remain in the oral cavity in elderly. In this context, root caries is an important problem [Tan et al., 2010; Walls and Meurman, 2012] because the challenge will be to maintain both the coronal and root caries under control.

Whenever the root is exposed to the oral cavity, enamel and cervical dentine are subjected to a similar environment: biofilm formation, dietary carbohydrates exposure, and fluoride use. Nevertheless, caries progress is faster in dentine than in enamel [Ogaard et al., 1988] because dentine has a higher organic matrix percentage [Kawasaki and Featherstone, 1997], higher permeability [ten Cate et al., 1998], and smaller crystals with higher carbonate concentration [Nyvad and Fejerskov, 1982], resulting in a higher critical pH for dentine than for enamel demineralization [Hoppenbrouwers et al., 1986; Wefel, 1994; Shellis, 2010]. Therefore, under the same cariogenic challenge, dentine should be considered more susceptible to demineralization than enamel.

Fluoride is still the main strategy for non-invasive control of root caries [Wierichs and Meyer-Lueckel, 2014]. Among all methods of fluoride delivery, toothpaste is responsible for the decline in enamel caries [Bratthall et al., 1996; Cury et al., 2004] and its recommendation to control enamel caries is based on strong evidence [Marinho et al., 2003; Walsh et al., 2010]. However, the knowledge about the anti-caries effect of fluoride on dentine is scarce [Featherstone, 1994; ten Cate et al., 1998], and some studies suggest that it would not be of the same magnitude as that on enamel [Herkstroter et al., 1991; ten Cate, 1999]. For dentine, higher fluoride concentration [ten Cate et al., 1995; Baysan et al., 2001; Mukai et al., 2001; Ekstrand et al., 2013; Srinivasan et al., 2014; Yeung, 2014], higher frequency of use [Laheij et al., 2010], or combination of methods of fluoride delivery [Vale et al., 2011] should be necessary to control caries; however, the evidence is not conclusive.

Models are widely used to evaluate the anti-caries potential of toothpastes; however, they should show dose-response effects because there is evidence that the effect of fluoride toothpaste

on caries control is concentration dependent [Walsh et al., 2010; Santos et al., 2013]. The most used models are chemical, named pH-cycling models, which simulate the caries process. pH-cycling models have been validated to evaluate the dose-response effect of standard and low fluoride toothpaste concentrations on enamel [Tenuta and Cury, 2013]. However, neither any validated model to evaluate the effect of fluoride on dentine nor on both enamel and dentine exists. Furthermore, pH-cycling models are chemical models that are not able to estimate the antimicrobial effect of fluoride or other substances on caries. Thus, biofilm models are more suitable to evaluate the relevance of antimicrobial effects on caries.

Biofilm models should mimic bacterial accumulation on dental surfaces and its exposure to a cariogenic challenge, thereby simulating the caries process. Further, these models should show a dose-response effect to antimicrobial exposure. We recently validated a *Streptococcus mutans* biofilm model to evaluate the effect of antimicrobial agents on biofilm formation and enamel demineralization [Ccahuana-Vásquez and Cury, 2010], using chlorhexidine as the positive control. This model was successfully used to evaluate the effect of iron on enamel [Ribeiro et al., 2012] and dietary products on either enamel and dentine demineralization [Giacaman et al., 2012; Munoz-Sandoval et al., 2012; Giacaman et al., 2014]. However, this model was not validated to evaluate the dose-response effect of fluoride either in enamel or dentine or simultaneously in both. Therefore, the aim of this study was to validate a cariogenic biofilm model to evaluate the effect of fluoride on enamel and root dentine under simultaneous conditions of demineralization.

MATERIALS AND METHODS:

Experimental design

This study was approved by the local Research and Ethics Committee (protocol No.108/2011). The current biofilm model was adapted from the Ccahuana-Vasquez and Cury's model [Ccahuana-Vásquez and Cury, 2010], which was previously validated to evaluate the antimicrobial agents. This model allows to estimate the anti-caries effect of substances on enamel and dentine demineralization inhibition upon a high cariogenic challenge, and also to analyze the

formed biofilm. *S. mutans* (SM) biofilms were formed on bovine enamel and root dentine slabs for 96 h. Biofilms/slabs were exposed 8×/day to 10% sucrose and 2×/day to 0, 150, 450, or 1,350 ppm F. These concentrations simulate the dilution (1:3) by saliva in the oral cavity during tooth brushing with fluoride toothpaste [Duke and Forward, 1982]; thus, simulating the use of toothpaste from low to high fluoride concentration (0, 500, 1,100, and 5,000 ppm F). The solutions were made with NaF and purified water. The medium was changed 2×/day and aliquots were analyzed to determinate the pH, F, and Ca concentration. At the end of experiment, the concentrations of water-soluble fluoride and acid-soluble fluoride were determined in the biofilms. In the slabs, the percentage of surface hardness loss (%SHL) and fluoride uptake were assessed. The study was conducted in three independent assays (n = 12/substrate/group) and response variables were blindly analyzed.

Enamel and root dentine slabs preparation

Slabs, 7 × 4 × 1 mm in size, were obtained from the crown and cervical roots of bovine incisors [Hara et al., 2003]. Enamel slabs were obtained from the central part of the dental crown [Ccahuana-Vásquez and Cury, 2010]. For root dentine, a 7 mm root slice was cut using two parallel diamond disks from the cementum-enamel junction, then cut to a 4 mm size mesiodistally to obtain the slab. Both substrates were flattened externally and internally on both surfaces, and the external surface was polished using 400, 600, and 1,200 grades of Al_2O_3 papers and polishing cloths with 1 µm diamond paste. The initial SH of slabs was determined by three indentations spaced 100 µm apart made with 50-g load for enamel and 5-g for root dentine on the polished surface, for 5 seconds (Future-Tech FM, Kawasaki, Japan). Slabs with a hardness of 336.2 ± 14.5 Kg/mm^2 (n = 48) for enamel and 36.5 ± 1.7 Kg/mm^2 (n = 48) for root dentine were included in the experiments. Ethylene oxide was used for sterilization of the slabs [Thomas et al., 2007].

Biofilm model

S. mutans UA159 (SM UA 159) biofilms were grown for 96 h on saliva-coated slabs of enamel and root dentine with surface hardness (SH) previously determined. One slab of each dental

substrate was assembled vertically using metallic holders in the same well of a 24-well culture plate. The slabs were immersed in filtered human saliva to simulate the formation of an acquired pellicle [Koo et al., 2003]. After washing in buffer solution, the slabs were transferred to culture plates containing 2 mL of ultrafiltered tryptone-yeast extract (LMW) broth with 1% sucrose and SM UA159 for initial bacterial adhesion. The inoculum was prepared from an exponential-growth culture of SM UA159 (100 µL of inoculum with the optical density of 1.6 was mixed with 50 mL of medium). After 8 h at 37°C, 10% CO_2 the slabs were transferred to fresh LMW containing 1 mM glucose, where they were kept overnight. Over the next three days after the adhesion phase, biofilms were exposed 8×/day (8:00, 9:30, 11:00, 12:00, 13:30, 15:00, 16:00, and 17:30 h) to 10% sucrose solution for 3 min. Twice a day, after the first and last sucrose exposure, biofilms were treated with the assigned fluoridated solutions for 1 min and rinsed three times in 0.9% NaCl. LMW (supplemented with 1 mM glucose) was changed twice per day to fresh media, before the first and after the second fluoride treatment. The 8×/daily sucrose exposure and the night immersion in fresh medium induced demineralization periods of continuous pH drops followed by at night remineralization periods at neutral pH. The pH was measured to estimate biofilm acidogenicity at each change of medium as well as F and Ca concentrations. After 96 h, biofilm formed on enamel and root dentine slabs were collected separately and analyzed for wet biofilm weight and soluble and bound-F concentration. Slabs surface hardness loss (%SHL) was evaluated as demineralization indicator. Fluoride uptake by enamel and root dentine was also determined as indicator of fluoride effect on the process of de- and remineralization.

Biofilm harvesting and analysis

At 96 h of biofilm growth and in the morning of the last overnight incubation, slabs were removed from the culture medium, washed three times in 0.9% NaCl and the slabs of enamel and dentine were individually immersed in 1 mL 0.9% NaCl placed in pre-weighed microcentrifuge tubes. To detach biofilm from slabs, the tubes were sonicated for 30 s at 7 W (Branson, Sonifier 50, Danburry, Conn., USA) and slabs were removed to carry out the demineralization analysis [Ccahuana-Vásquez and Cury, 2010]. Biofilm suspension was centrifuged at 10,000 *g*, 5 min at 4°C and the supernatant was collected for water soluble F analysis. The tube was again centrifuged

and remnants of the supernatant was carefully vacuum-aspirated with a micropipette under a microscope to remove any solution. The biofilm pellet was weighed to obtain biomass (wet weight) and frozen for further analysis of bound F.

Culture medium analyses

The medium collected twice/day (after the daily de- and remineralization periods) was analyzed for pH, and F and Ca concentration. Medium pH was determined using a microelectrode (Cole-Parmer Accumet, Vernon Hills, IL, USA) coupled to a pH meter (Procyon SA-720, Olímpia, SP, Brazil). For fluoride analysis, aliquots were buffered using TISAB II (1:1) and analyzed with an ion-selective electrode (Orion 96-09; Orion Research) and an ion analyzer (Orion EA-940; Orion Research), which had been previously calibrated with standard fluoride solutions prepared similarly to the samples. Ca was analyzed by colorimetric analysis using Arsenazo III [Vogel et al., 1983], and the absorbance was read at 650 nm in 96-well microplates using a Multiskan Spectrum (Thermo Scientific) microplate reader.

Determination of water soluble and bound F in biofilms

Soluble fluoride was measured in the supernatant of the saline extract. Aliquots were buffered using TISAB II (1:1) and analyzed with specific electrode as described above.

Bound fluoride was extracted from the biofilm pellet by treatment with 0.5 M HCl (0.15 mL/10 mg bacterial wet weight) for 3 h [Cury et al., 1997]. The extract was centrifuged for 3 min at 16,000 *g* and F concentration in the supernatant was determined using a fluoride electrode adapted for microanalysis, after neutralization with 2.5 M NaOH and buffering with TISAB III [Vogel et al., 1983]. For the analyses, the microelectrode was previously calibrated with F standards prepared as the samples.

Demineralization determination

SH was used as an indicator of enamel [Cury et al., 2000] and dentine [Vale et al., 2011] demineralization. Three indentations, 100 µm apart, were made on the substrates before and after

each experimental phase. Demineralization was expressed as percentage of surface hardness loss (%SHL) and calculated by the formula (baseline SH–SH after treatment)/baseline SH*100.

Determination of F in enamel and dentine

The surfaces of enamel and root dentine slabs were isolated with wax, except the external surface where the indentations were made. The area exposed was determined and the slabs were immersed in 0.5 M HCl (3.57 mL/cm²) for 30 s under constant agitation (150 rpm) to remove an enamel or root dentine layer. The extract was buffered with an equal volume of TISAB II (pH 5.0), modified with 20 g NaOH/L [Koo and Cury, 1998]. The F concentration was determined with a specific F electrode as described for soluble F determination. Pi was measured in the acid extract [Fiske and Subbarow, 1925] and the amount of enamel or dentine dissolved was calculated based on Pi concentration and density [Koo and Cury, 1998] of 17.4% and 2.92 g/cm^3 for enamel and 13.5% and 2.14 g/cm^3 for dentine. Fluoride concentration was expressed in µg F/g of enamel or dentine.

Statistical Analysis

Data were analyzed by two-way ANOVA, considering the factors substrate (enamel or dentine) and F concentration (0, 150, 450, or 1,350 ppm F) using SAS system (SAS Institute Inc., version 9.2, Cary, NC, USA). Assumptions of homogeneity of variances and normal distribution of errors were checked for all response variables tested, and variables that did not satisfy these assumptions were transformed as suggested by the software [Box et al., 1978]. Regression analyses between %SHL and F concentration were also calculated. The significance level was set at 5%.

RESULTS

The pH value of the culture medium, used as an indicator of biofilm acidogenicity, decreased after the daily sucrose exposure but differences among the treatments were not observed (data not shown). F and Ca concentration in the culture medium (Figure 1) showed distinct patterns with fluoride concentration of the treatments, i.e., while fluoride increased, calcium decreased.

Regarding soluble fluoride concentration in biofilms, formed either on enamel or on dentine, the lowest and highest values ($p < 0.05$) were found for the negative control group and the treatment group with greater fluoride concentration, respectively (Table 1). However, for bound fluoride the difference among fluoride groups was not significant ($p > 0.05$) and only the biofilms formed on dentine differed from the non-fluoride group. Fluoride concentrations in biofilm formed on dentine by fluoride treatments were higher than those on enamel and statistically significant for the treatments with the highest fluoride concentration (Table 1).

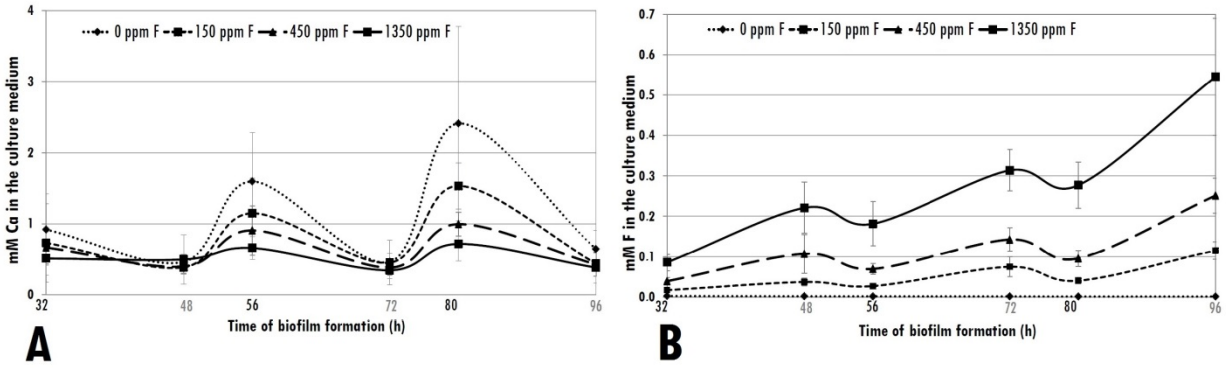


Figure 1: Calcium (A) and fluoride (B) concentration (mM) in the culture medium after cariogenic challenge period on the second day (32 h), third day (56 h), and on fourth day (80 h) and after the overnight period on the second day (48 h), third day (72 h), and on fourth day (96 h) (mean ± SD; n = 12).

Table 1. Soluble and bound fluoride in biofilms (µmol F/g) formed on enamel and dentine slabs, after treatment with various fluoride concentrations (mean ± SD; n = 12).

Treatments[α]	Soluble F [β]		Bound F	
(ppm F)	Enamel	Dentine	Enamel	Dentine
0 (control)	0.33±0.07 A,a	[γ]0.30±0.09 A,a	0.17±0.08 A,a	0.12±0.07 B,a
150	1.93±0.49 A,b	2.25±0.39 A,b	0.22±0.09 A,a	0.31±0.31 A,b
450	2.89±0.54 A,c	4.37±1.03 B,c	0.23±0.07 A,a	0.32±0.15 A,b
1,350	4.65±1.07 A,d	7.63±1.53 B,d	0.28±0.11 A,a	0.46±0.30 B,b

Distinct capital letters indicate differences ($p < 0.05$) between enamel and dentine for soluble and bound fluoride (values within lines).
Distinct lower case letters indicate differences ($p < 0.05$) among treatments (values in columns for each condition) .
[α] Concentrations to simulate brushing with toothpaste from low to high F concentration, considering the dilution (1:3) by saliva
[β] values transformed by log 10; [γ] outlier removed of group, value = 0.74 mol F/g biofilm.

In terms of the effect of the treatments on enamel and dentine demineralization (Table 2), a dose-response effect was seen for both dental substrates with a negative linear relationship between %SHL and fluoride concentrations of the treatments. Highest demineralization ($p < 0.05$) was found for the non-fluoride treatment group, either for enamel or dentine. In addition, demineralization was consistently higher in dentine than in enamel ($p < 0.05$) for all groups (Table 2). Thus, assuming the effect of fluoride on dentine was equivalent to that on enamel, dentine would have be treated with fluoride concentration 3× higher than enamel, 1,350 vs. 450 ppm F, as shown in Table 3.

Table 2. Percentage of surface hardness loss (%SHL) of enamel and dentine, after treatment with various fluoride concentrations (mean ± SD; n = 12) and regression analysis values.

Treatments[α] (ppm F)	Surface hardness loss (%SHL)			
	Enamel[β]		Dentine	
0 (control)	29.2±8.6	A,a	49.2±4.8	B,a
150	15.5±5.8	A,b	38.7±2.5	B,b
450	11.4±4.7	A,bc	31.0±5.9	B,c
1,350	6.9±3.0	A,c	20.3±6.0	B,d
Linear regression	r=-0.727; p<0.001		r=-0.852; p<0.001	

Distinct capital letters indicate differences ($p < 0.05$) between enamel and dentine (values within lines)
Distinct lower case letters indicate differences ($p < 0.05$) among fluoride treatments (values in columns for enamel and dentine
[α] Concentrations to simulate brushing with toothpaste from low to high F concentration, considering the dilution (1:3) by saliva
[β] values transformed by log 10

Table 3. Reduction (%) of enamel and root dentine demineralization after treatment with various fluoride concentrations compared with their respective controls.

Treatment[α] (ppm F)	*Reduction of demineralization(%)	
	Enamel vs control	Dentine vs control
0 (control)	-	-
150	46.8	21.3
450	60.9**	36.9
1,350	76.1	58.8**

*Relative to control
**Similar effect, about 60%, is observed with 450 ppm F for enamel and with 1,350 ppm F for dentine
[α] Concentrations to simulate brushing with toothpaste from low to high F concentration, considering the dilution (1:3) by saliva

The data on fluoride concentration found in enamel and dentine (Table 4) were coherent with those observed for demineralization (Table 2). A dose-response effect was observed for both dental substrates and higher fluoride concentration was found in dentine than enamel ($p < 0.05$) (Table 4).

Table 4. Fluoride concentration (µg F/g) in enamel and dentine after treatment with various fluoride concentrations (mean ± SD; n = 12) and values of regression analysis.

Treatments[α] (ppm F)	Fluoride concentration (µg F/g) [β]			
	Enamel		Dentine	
0 (control)	256.4±65.8	A,a	1105±331	B,a
150	439.4±200.0	A,b	3896±964	B,b
450	588.2±223.4	A,bc	4697±1113	B,bc
1,350	760.7±241.9	A,c	6133.±1280	B,c
Linear regression	r = 0.665; p < 0.001		r = 0.688p; p < 0.001	

Distinct capital letters indicate differences ($p < 0.05$) between enamel and dentine (values within lines)
Distinct lower case letters indicate differences ($p < 0.05$) among fluoride reatments (values in columns for enamel and dentine
[α] Concentrations to simulate brushing with toothpaste from low to high F concentration, considering the dilution (1:3) by saliva
[β] values transformed by log 10

DISCUSSION

This study was conducted because of the absence of studies on a validated cariogenic biofilm model to evaluate the dose-response effect of fluoride either on enamel or dentine demineralization, or simultaneously in both. The model was validated, and it enabled assessment of the anti-caries effect of fluoride on demineralization of dental substrates and also analyze the effect of treatments on the biofilm.

The dose-response effect of fluoride on reduction of demineralization was found for enamel and dentine (Table 2). The concentrations of 150–1,350 ppm F used in the experiment, simulates the dilution 1:3 that occurs in the oral cavity [Duke and Forward, 1982] when toothbrushing with toothpastes of low to high fluoride concentration. Therefore, this model could be used to estimate the anti-caries potential of innovative toothpaste formulations with low

fluoride concentration [Negri and Cury, 2002] that could be recommended to control enamel caries in children, and formulations with high fluoride concentrations, that have been suggested to control root caries in elderly people [Baysan et al., 2001; Ekstrand et al., 2013; Srinivasan et al., 2014]. Furthermore, the model could be used to estimate the anti-caries potential of fluoride on the cervical area in adults and elderly people, where dentine is exposed and enamel and dentine are at the same risk of caries.

Although a linear dose-response effect of fluoride concentration on reduction of demineralization has been found for enamel and dentine, the data clearly showed that the effect of fluoride was different on these dental substrates (Table 3). Thus, while 450 ppm F produced a 60% reduction in demineralization of enamel, in dentine this effect at the same percentage could only be obtained with 1,350 ppm F. These findings can be explained by the fact that dentine is considered more susceptible to caries than enamel [Nyvad and Fejerskov, 1982; Wefel, 1994] and moreover we found approximately twice more demineralization in dentine (49%) than in enamel (29%) not treated with fluoride, i.e., negative control group as shown in Table 2. Our findings suggest that 3× more fluoride concentration would be necessary for dentine in order to achieve the same effect on percentage of reduction of demineralization as in enamel, which support clinical data suggesting that more fluoride would be required to control root caries [Baysan et al., 2001; Ekstrand et al., 2013; Srinivasan et al., 2014] than that used for enamel. Also, the difference of fluoride effect on dentine compared with enamel explains why the combination of professional fluoride application with daily regular use of standard fluoride toothpaste would be more effective to control caries in dentine [Vale et al., 2011] but not in enamel [Marinho et al., 2004; Paes Leme et al., 2004]. Contrary to enamel, on dentine the effect of the professional application in combination with daily fluoride toothpaste use was synergistic compared with the isolated effects of the two methods of fluoride application.

The dose-response effect of fluoride concentration on reduction of enamel and dentine demineralization, evaluated by surface hardness (Table 2) is also supported by the Ca concentration in the culture medium (Figure 1A). Ca is a chemical indicator of enamel and dentine demineralization and its concentration in the medium after the 8 sucrose exposures (demineralization period, times of 32, 56, and 80 h) was higher in the control group, where greater

%SHL was found. Calcium concentrations were proportionally lower in the groups treated with fluoride. After the overnight remineralization period, when the biofilm stayed immerse in neutral medium for 48, 72, and 96 h, the difference between the groups decreased but the trend was maintained (Figure 1A). The results from fluoride concentration in the medium (Figure 1B) showed a pattern different from Ca and reflects the concentration of fluoride treatments.

The data of soluble fluoride found in biofilm (Table 1) also give support to the dose-response effect of fluoride concentration on demineralization in either enamel or dentine (Table 2). This concentration reflects the effect of the last treatment with fluoride because the biofilms were collected after the overnight remineralization period. This concentration also reflects the concentration found in the culture medium (Figure 1B). Concentrations of soluble fluoride in the biofilm was directly proportional to fluoride concentration of the treatments and help to explain the effect of fluoride on enhancement of remineralization.

We also found a dose-response effect between fluoride concentration of the treatments and fluoride concentration either in enamel or dentine (Table 4). This concentration of fluoride in enamel and dentine is a consequence of the mechanism of fluoride on the caries process. Fluoride interferes physicochemically with the caries process, reducing the demineralization of enamel and dentine when the pH falls after biofilm exposure to sugar and enhancing the remineralization process when pH rises again above the critical. Our model simulated the caries process because the biofilms were treated 8×/day with sucrose, when enamel and dentine were simultaneous subjected to demineralization, and during the night the slabs were subjected to remineralization because the biofilms were not treated with sucrose, and the pH was maintained around neutral values. Enamel and dentine were enriched with fluoride due to precipitation of fluoride apatite in enamel and dentine during the de-remineralizing process [Cury and Tenuta, 2009]. It is well known that dentine is more reactive to fluoride than enamel [ten Cate et al., 1995] but our findings show that dentine also gains more fluoride than enamel during the dynamics of the caries process (Table 4). The present data extend those found by ten Cate et al. (1995), who showed that carious dentine incorporates more fluoride than enamel when treated with fluoride toothpaste and subjected to a chemical pH-cycling model. This higher amount of fluoride uptake in dentine during the caries process could be explained by two mechanisms: first, it would be a simple consequence by the fact

that dentine is more demineralized than enamel (Table 2) and consequently more fluoride are changed with the minerals dissolved from dentine. Second, dentine has more amorphous calcium phosphate minerals than enamel and fluoride activates the phase transformation of these minerals in fluorapatite [Moreno and Zahradnik, 1974; Wefel, 1994].

The effect of fluoride found in the present study, reducing demineralization either in enamel or dentine, was essentially physicochemical, as described above. In fact, we did not find an effect of fluoride concentration on biofilm weight and acidogenicity. Fluoride may have antimicrobial effects but it is concentration dependent and fluoride concentration either in biofilm treated with the greater concentration used (Table 1) or in the culture medium (Fig 1B) were below 10 ppm, the minimum fluoride concentration to inhibit enolase [Bradshaw et al., 2002]. The findings gives support to the knowledge that the mechanism of action of fluoride on caries control is local and the antimicrobial effect of fluoride may be marginal when compared with the physicochemical effect [Cury and Tenuta, 2008; Tenuta and Cury, 2010].

In summary, the findings consistently showed that this biofilm model is valid to evaluate the effect of fluoride in either enamel or dentine demineralization, or simultaneously in both. It should be emphasized that this model was validated in terms of dose-response effect of fluoride concentration to estimate the anti-caries potential of toothpaste formulations but it could also be useful in testing mouth rinse formulations. However, it was not validated to test methods of professional fluoride application or fluoride releasing materials.

ACKNOWLEDGMENTS

This study is based on the first author's doctorate thesis (Graduate Program in Dentistry, Cariology Area, Faculty of Dentistry of Piracicaba, State University of Campinas, UNICAMP, Brazil). The first author received a scholarship during this study by Government of Chile, National Commission for Scientific and Technological Research (CONICYT), BECAS CHILE. Res.1166/2011. Preliminary reports were presented at the 91th General Session of the IADR, Seattle, USA, 2013 and the 30th Annual Meeting of SBPqO (Brazilian Society of Dental Research), Brazil, 2013. Conceived and designed the experiment: JC. Improved the experimental design and performed the experiment: CF. Analyzed the data: CF, JC, and LT. Wrote the paper: CF, JC, and LT.

REFERENCES

Baysan A, Lynch E, Ellwood R, Davies R, Petersson L, Borsboom P: Reversal of primary root caries using dentifrices containing 5,000 and 1,100 ppm fluoride. Caries Res 2001;35:41-46.

Box G, Hunter W, Hunter J: Statistics for experimenters: An introduction to design, data analysis, and model building. New York, John Wiley & Sons Inc., 1978.

Bradshaw DJ, Marsh PD, Hodgson RJ, Visser JM: Effects of glucose and fluoride on competition and metabolism within in vitro dental bacterial communities and biofilms. Caries Res 2002;36:81-86.

Bratthall D, Hansel-Petersson G, Sundberg H: Reasons for the caries decline: What do the experts believe? Eur J Oral Sci 1996;104:416-422; discussion 423-415, 430-412.

Ccahuana-Vásquez RA, Cury JA: S. Mutans biofilm model to evaluate antimicrobial substances and enamel demineralization. Braz Oral Res 2010;24:135-141.

Cury JA, Tenuta LM: Enamel remineralization: Controlling the caries disease or treating early caries lesions? Braz Oral Res 2009;23 Suppl 1:23-30.

Cury JA, Tenuta LM: How to maintain a cariostatic fluoride concentration in the oral environment. Adv Dent Res 2008;20:13-16.

Cury JA, Tenuta LM, Ribeiro CC, Paes Leme AF: The importance of fluoride dentifrices to the current dental caries prevalence in brazil. Braz Dent J 2004;15:167-174.

Cury JA, Rebelo MA, Del Bel Cury AA, Derbyshire MT, Tabchoury CP: Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res 2000;34:491-497.

Cury JA, Rebello MA, Del Bel Cury AA: In situ relationship between sucrose exposure and the composition of dental plaque. Caries Res 1997;31:356-360.

Duke SA, Forward GC: The conditions occurring in vivo when brushing with toothpastes. Br Dent J 1982;152:52-54.

Ekstrand KR, Poulsen JE, Hede B, Twetman S, Qvist V, Ellwood RP: A randomized clinical trial of the anti-caries efficacy of 5,000 compared to 1,450 ppm fluoridated toothpaste on root caries lesions in elderly disabled nursing home residents. Caries Res 2013;47:391-398.

Featherstone JD: Fluoride, remineralization and root caries. Am J Dent 1994;7:271-274.

Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. Journal of Biological Chemistry 1925;66:375-400.

Giacaman RA, Contzen MP, Yuri JA, Munoz-Sandoval C: Anticaries effect of an antioxidant-rich apple concentrate on enamel in an experimental biofilm-demineralization model. J Appl Microbiol 2014. Sep;117(3):846-853

Giacaman RA, Munoz MJ, Ccahuana-Vasquez RA, Munoz-Sandoval C, Cury JA: Effect of fluoridated milk on enamel and root dentin demineralization evaluated by a biofilm caries model. Caries Res 2012;46:460-466.

Herkstroter FM, Witjes M, Arends J: Demineralization of human dentine compared with enamel in a ph-cycling apparatus with a constant composition during de- and remineralization periods. Caries Res 1991;25:317-322.

Hoppenbrouwers PM, Driessens FC, Borggreven JM: The vulnerability of unexposed human dental roots to demineralization. J Dent Res 1986;65:955-958.

Kawasaki K, Featherstone JD: Effects of collagenase on root demineralization. J Dent Res 1997;76:588-595.

Koo H, Hayacibara MF, Schobel BD, Cury JA, Rosalen PL, Park YK, Vacca-Smith AM, Bowen WH: Inhibition of streptococcus mutans biofilm accumulation and polysaccharide production by apigenin and tt-farnesol. J Antimicrob Chemother 2003;52:782-789.

Koo RH, Cury JA: Soluble calcium/smfp dentifrice: Effect on enamel fluoride uptake and remineralization. Am J Dent 1998;11:173-176.

Laheij AM, van Strijp AJ, van Loveren C: In situ remineralisation of enamel and dentin after the use of an amine fluoride mouthrinse in addition to twice daily brushings with amine fluoride toothpaste. Caries Res 2010;44:260-266.

Marinho VC, Higgins JP, Sheiham A, Logan S: Combinations of topical fluoride (toothpastes, mouthrinses, gels, varnishes) versus single topical fluoride for preventing dental caries in children and adolescents. Cochrane Database Syst Rev 2004:CD002781.

Marinho VC, Higgins JP, Sheiham A, Logan S: Fluoride toothpastes for preventing dental caries in children and adolescents. Cochrane Database Syst Rev 2003:CD002278.

Moreno EC, Zahradnik RT: Chemistry of enamel subsurface demineralization in vitro. J Dent Res 1974;53:226-235.

Mukai Y, Lagerweij MD, ten Cate JM: Effect of a solution with high fluoride concentration on remineralization of shallow and deep root surface caries in vitro. Caries Res 2001;35:317-324.

Munoz-Sandoval C, Munoz-Cifuentes MJ, Giacaman RA, Ccahuana-Vasquez RA, Cury JA: Effect of bovine milk on streptococcus mutans biofilm cariogenic properties and enamel and dentin demineralization. Pediatr Dent 2012;34:e197-201.

Negri HM, Cury JA: [dose-response effect of a dentifrice formulation with low fluoride concentration - an in vitro study]. Pesqui Odontol Bras 2002;16:361-365.

Nyvad B, Fejerskov O: Root surface caries: Clinical, histopathological and microbiological features and clinical implications. Int Dent J 1982;32:311-326.

Ogaard B, Rolla G, Arends J: In vivo progress of enamel and root surface lesions under plaque as a function of time. Caries Res 1988;22:302-305.

Paes Leme AF, Dalcico R, Tabchoury CP, Del Bel Cury AA, Rosalen PL, Cury JA: In situ effect of frequent sucrose exposure on enamel demineralization and on plaque composition after apf application and f dentifrice use. J Dent Res 2004;83:71-75.

Petersen PE, Yamamoto T: Improving the oral health of older people: The approach of the who global oral health programme. Community Dent Oral Epidemiol 2005;33:81-92.

Ribeiro CC, Ccahuana-Vasquez RA, Carmo CD, Alves CM, Leitao TJ, Vidotti LR, Cury JA: The effect of iron on streptococcus mutans biofilm and on enamel demineralization. Braz Oral Res 2012;26:300-305.

Santos AP, Oliveira BH, Nadanovsky P: Effects of low and standard fluoride toothpastes on caries and fluorosis: Systematic review and meta-analysis. Caries Res 2013;47:382-390.

Shellis RP: Formation of caries-like lesions in vitro on the root surfaces of human teeth in solutions simulating plaque fluid. Caries Res 2010;44:380-389.

Srinivasan M, Schimmel M, Riesen M, Ilgner A, Wicht MJ, Warncke M, Ellwood RP, Nitschke I, Muller F, Noack MJ: High-fluoride toothpaste: A multicenter randomized controlled trial in adults. Community Dent Oral Epidemiol 2014;42:333-340.

Tan HP, Lo EC, Dyson JE, Luo Y, Corbet EF: A randomized trial on root caries prevention in elders. J Dent Res 2010;89:1086-1090.

ten Cate JM: Current concepts on the theories of the mechanism of action of fluoride. Acta Odontol Scand 1999;57:325-329.

ten Cate JM, Damen JJ, Buijs MJ: Inhibition of dentin demineralization by fluoride in vitro. Caries Res 1998;32:141-147.

ten Cate JM, Buijs MJ, Damen JJ: Ph-cycling of enamel and dentin lesions in the presence of low concentrations of fluoride. Eur J Oral Sci 1995;103:362-367.

Tenuta LM, Cury JA: Laboratory and human studies to estimate anticaries efficacy of fluoride toothpastes. Monogr Oral Sci 2013;23:108-124.

Tenuta LM, Cury JA: Fluoride: Its role in dentistry. Braz Oral Res 2010;24 Suppl 1:9-17.

Thomas RZ, Ruben JL, ten Bosch JJ, Huysmans MC: Effect of ethylene oxide sterilization on enamel and dentin demineralization in vitro. J Dent 2007;35:547-551.

Vale GC, Tabchoury CP, Del Bel Cury AA, Tenuta LM, ten Cate JM, Cury JA: Apf and dentifrice effect on root dentin demineralization and biofilm. J Dent Res 2011;90:77-81.

Vogel GL, Chow LC, Brown WE: A microanalytical procedure for the determination of calcium, phosphate and fluoride in enamel biopsy samples. Caries Res 1983;17:23-31.

Walls AW, Meurman JH: Approaches to caries prevention and therapy in the elderly. Adv Dent Res 2012;24:36-40.

Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X: Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev 2010:CD007868.

Wefel JS: Root caries histopathology and chemistry. Am J Dent 1994;7:261-265.

Wierichs RJ, Meyer-Lueckel H: Systematic review on noninvasive treatment of root caries lesions. J Dent Res 2014.

Yeung CA: Some beneficial effect on root caries from use of higher concentration fluoride toothpaste (5000 ppm f). Evid Based Dent 2014;15:8-9.

CAPÍTULO 2

5,000 ppm-F-dentifrice or 1,100 plus APF on enamel-dentine de-remineralization

C.E. Fernández; D.F. Nóbrega; L.M.A. Tenuta; A.A. Del Bel Cury; J.A. Cury

Piracicaba Dental School, UNICAMP, Piracicaba, São Paulo, Brazil.

Corresponding author:

Jaime A Cury

Piracicaba Dental School, UNICAMP

Av. Limeira, 901. Piracicaba SP 13414-903

Brazil

Tel/Fax. +55 19 21065302

E-mail jcury@fop.unicamp.br

ABSTRACT

The effect of fluoride dentifrice (FD) on enamel demineralization-remineralization is concentration dependent, but there is scanty data about root dentine. Additionally, there is some evidence of a bigger effect of a high-FD (5,000-ppm-F) for root dentine than for enamel caries control, which may be equaled by the daily use of a standard concentration FD (1,100-ppm-F) in combination with professional fluoride application. Therefore, we hypothesized that the effect of FD on de-remineralization of enamel or root-dentine is dose-dependent, but the combination APF+FD-1,100 would have greater effect on dentine caries control than a high-FD. An *in situ*, double-blind and crossover study was conducted in 4 phases of 14 days each, during which 18 volunteers wore palatal appliances containing enamel and root-dentine slabs, either sound or carious to evaluate the effect of the treatments on de-remineralization, respectively. The four treatment groups were: placebo-FD use (control), 1,100 or 5,000-FD, used twice/day, or previous application on slabs of acidulated phosphate fluoride (APF-gel; 12,300-ppm-F) combined with daily use of 1,100-FD (APF+1,100-FD). Fluoride in biofilm was determined; in the slabs, de-remineralization were assessed by surface and cross-sectional hardness and alkali and acid-soluble-fluoride concentration were determined. The dose-response for FD-concentration, analyzed by linear regression, was significant (0;1,100;5,000; $p<0.05$) for most variables analyzed, either for enamel or dentine. ANOVA showed that, for dentine, the combination of APF+1,100-FD was significantly more effective than 5000-FD on reduction of demineralization and enhancement of remineralization ($p<0.05$), in agreement with data of alkali-soluble-fluoride concentration found on slabs. For enamel, no significant difference between APF+1,100-FD and 5,000-FD was found, in consistency with the overall results for this substrate. The findings suggest that either the inhibition of demineralization or enhancement of remineralization of either enamel or root-dentine is FD-concentration dependent but, the combination of APF+1,100-FD would be relevant only to control root-dentine caries.

Keywords: toothpaste, fluorides, tooth demineralization, calcium fluoride, topical fluorides, acidulated phosphate fluoride.

INTRODUCTION

Dental caries is a dynamic process and caries lesions can be reduced, reverted or arrested (Kidd and Fejerskov, 2013). Fluoride has an important effect in the process of caries development, through its physicochemical action reducing demineralization (de-) and enhancing remineralization (re-) when respectively, the pH in biofilm fluid falls after sugar exposure and rises again (Cury and Tenuta, 2008; ten Cate, 2013). Under a high caries challenge (de->re-) fluoride reduces the net demineralization that occurs in sound teeth ('preventive effect') but when the caries challenge is low (re->de-), fluoride enhances saliva remineralization property and caries lesions already present can be repaired ('therapeutic effect'). Not only the relative effect of fluoride on enamel or dentine, but also its effect on the reduction of demineralization or enhancement of remineralization to which these dental substrates are subjected, are still under study, especially in root-dentine.

Among the available ways of fluoride use, toothbrushing with fluoride dentifrice (FD) has been considered the most promising strategy to control crown and root caries (Cury and Tenuta, 2014; Kidd and Fejerskov, 2013). However, while the effect of fluoride dentifrice (FD) on enamel caries is strongly based on evidence (Marinho et al., 2003) and concentration-dependent (Walsh et al., 2010), there is very few data about its effect on root dentine. The control of root caries is challenging due to the increased life expectation, greater number of teeth and surfaces exposed (Walls and Meurman, 2012) and mainly the greater dentine susceptibility to caries when compared to enamel (Nyvad and Fejerskov, 1982). Also, in terms of caries, enamel and dentine cannot be considered as independent tissues because gingival recession simultaneously subjects both to the same environmental conditions, i.e. biofilm accumulation, sugar and/or fluoride (F) exposure. Furthermore, it has been suggested that the strength of F effect on dentine would not be the same than on enamel (Heijnsbroek et al., 2007; Petersson, 2013; ten Cate et al., 1998).

Therefore, for a more effective root caries control, the use of additional F treatments has been recommended (Petersson, 2013; Wierichs and Meyer-Lueckel, 2014). Also, there is some evidence that toothbrushing with high fluoride dentifrice (5,000-FD) (Baysan et al., 2001; Ekstrand et al., 2013; Srinivasan et al., 2014) or the daily regular use of 1,100-FD combined with

professional fluoride application (Vale et al., 2011) should be recommended for a more effective control of root caries.

Nevertheless, no study until now evaluated the effect of fluoride on enamel and dentine when these dental substrates are simultaneously subjected to the same experimental conditions. Also, the effect of fluoride on these substrates, on either the reduction of demineralization, simulating a high caries-risk patient, or the enhancement of remineralization, simulating a low caries-risk patient, has not been evaluated. Therefore, we designed this *in situ* study to test the hypothesis that the effect of FD would be concentration dependent on either enamel or dentine de-remineralization, but the effect of combination with professional fluoride application (APF+1,100-FD) would have greater effect on dentine caries control than a high-FD.

MATERIAL AND METHODS

Ethical aspects and volunteers

This *in situ* study was approved by the local Research and Ethics Committee (protocol No.108/2011) and 18 volunteers, who fulfilled the inclusion criteria described by Botelho et al., (2014), signed a written informed consent for participation. They were healthy adults (24.1±4.01 years old), with a mean (±SD) DMFS index of 3.3±4.1.

Experimental Design

The study used an *in situ*, double-blind and crossover design, conducted in four phases of 14 days each. Volunteers wore palatal appliances containing two enamel and two root-dentine slabs on each side, either sound or carious. Sucrose was dropped 8x/day on sound-specimens to simulate high caries challenge (de->re-) or 3x/day on carious-specimens to simulate low caries challenge (re->de-). In each phase, groups of volunteers were subjected to one of the following treatments: F placebo dentifrice (PD), 1,100-FD, 5,000-FD or combination of APF (gel, 12,300 ppm F) applied once on slabs at the beginning of the experimental phase with 1,100-FD (APF+1,100-FD). Dentifrices were used 2x/day. PD was used during the pre-experimental and washout periods. At

the end of each phase, F concentration in the biofilm was assessed. The effect of treatments on reduction of demineralization, occurred on the originally sound slabs, and the enhancement of remineralization on the carious slabs was evaluated by surface and cross-sectional hardness. Also, alkali and acid-soluble-F-concentration were determined on slabs.

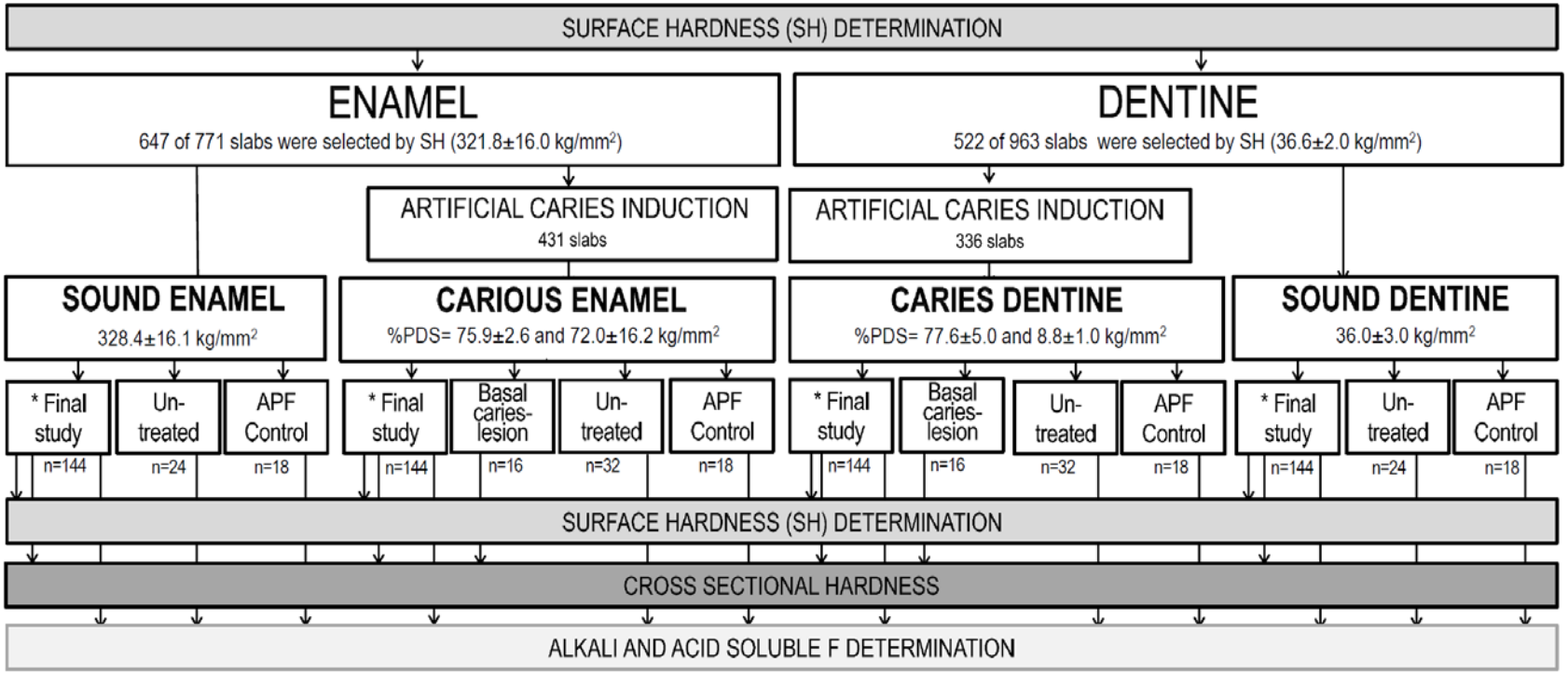
Slabs and palatal appliance preparation

Enamel and root-dentine slabs (4x4x2 mm) were prepared from bovine crown and root (Hara et al., 2003) and surface hardness (SH) was determined on enamel (Cury et al., 2000) and dentine (Vale et al., 2011). Root-dentine slabs were maintained 24 h in artificial saliva to allow root-dentine mineral gain and minimize further ionic changes when exposed to saliva *in situ* (Hara et al., 2003). In addition, before performing SH measurements, these slabs were allowed to dry for at least 30 minutes to standardize the measurements (Vale et al., 2011).

Enamel and dentine slabs were selected by SH and then randomized for sound or carious conditions studied (Figure 1). From these groups, 144 sound enamel and 144 sound dentine were selected for the *in situ* study. In the rest of enamel and dentine slabs, incipient sub-superficial (80 µm depth) caries lesions were induced in the rest of enamel and dentine slabs, using a solution 50% saturated regarding the solubility of the dental substrates (Moreno and Zahradnik, 1974). For enamel, the lesions were induced according to Queiroz et al., (2008). For root-dentine, the slabs were maintained 16 h at 37°C in 0.05 mol/L acetate buffer, pH 5, containing 1.4 mmol/L Ca, 0.91 mmol/L Pi and 0.06 µg F/mL. SH was again determined and the %SH loss was calculated (%SHL). Carious enamel and dentine slabs were selected (n=144 per group) for the final study (Figure 1). Slabs used *in situ* were allocated to the groups and phases by stratified randomization. Moreover, 18 slabs of enamel and dentine, sound and carious were selected for the evaluation of F formed by APF application (Figure 1).

Sound slabs (2 of enamel and 2 of dentine) were mounted in one side of each appliance whereas the carious ones in the other side. For half of the volunteers, the sequence of slabs started from the anterior to posterior with enamel (enamel-dentine-enamel-dentine) and for the other half with

Figure 1. Selection and distribution of enamel and dentine slabs used in the experiment



* *in situ* phases

dentine (dentine-enamel-dentine-enamel). Also, for half of the volunteers the sound ones were at the right side and for the other half at the left side of the appliance. This appliance setup was fixed for each volunteer at the different phases.

Treatments

The cariogenic challenges were provided by dripping a 20% sucrose solution onto slabs extra-orally (Cury et al., 2000). A plastic mesh was fixed 1 mm above slabs to encourage biofilm accumulation (Hara et al., 2003); red or colorless resin was used to identify sucrose drip frequency. For sound specimens sucrose was dripped 8x/day (8.00, 9.00, 10.00, 11.00, 14.00, 15.30, 17.00, 19.00 h); for carious specimens, sucrose was dripped 3x/day (8.00, 14.00, 19.00 h). After 5 min, the appliance was re-inserted in the mouth (Cury et al., 2000).

The dentifrices were specially formulated for this research by Colgate Palmolive from Brazil with identical formulations (NaF/silica), differing only on F concentration. The volunteers used twice/day (7.00 and 21.00 h) the assigned dentifrices for each phase and they were instructed how to use them, as described by Cury et al. (2010). PD was used during the pre-experimental and washout periods for at least 3 days (Fernández et al., 2015). No restrictions were imposed with regard to their diet, considering the crossover design of this study; volunteers had access to optimally fluoridated water (0.7 ppm F, ranging from 0.6 to 0.8).

For the combination group, APF (12,300 ppm F, pH = 3.6–3.9; DFL, Petropolis, RJ, Brazil) was applied on slabs as described previously (Vale et al., 2011), before fixing the mesh to the appliance. In addition, the F formed immediately after APF application was evaluated with extra slabs fixed in the appliance in an anterior position (Vale et al., 2011), which were removed immediately after the application for analysis. The appliance was remained in the mouth for 30 min to simulate the F clearance effect by saliva (Paes Leme et al., 2004), then the mesh was fixed (Hara et al., 2003) .

F analysis in the biofilm

After 14 days of experiment, biofilms formed on the slabs were collected at morning, 10 h after last toothbrushing. Biofilms were collected separately and pooled for each substrate and condition. The fluid was separated from the biofilm solids by centrifugation and immediately collected and analyzed as described previously (Tenuta et al., 2006; Vogel et al., 1997). The extraction of acid-soluble F in solid portion of biofilm was made after fluid extraction as described by Ccahuana-Vasquez et al. (2007). F concentration was measured using an inverted F electrode (Vogel et al., 1997).

Enamel and dentine analyses

At the end of each phase, one row of three adjacent indentations was made on slabs surface 100 μm from the three baseline measurements (when the initial indentations were not noticed, the load was made in the center of the slab). The percentage of SH loss and recovery were respectively calculated for the originally sound (Cury et al., 2000) and carious-specimens (Paes Leme et al., 2003). SH was used as indicator of mineral changes because it presents high correlation with transversal microradiography for enamel (White, 1987) and dentine (Vale et al., 2011). After SH analysis, the slabs were longitudinally sectioned on the center and one half was used to cross-sectional hardness (CSH) assessment and the other half was used to F analysis. The CSH analysis and the calculation of the caries lesion area (ΔS; $kg/mm^2 \times \mu m$) was made according to Calvo et al. (2012). The effect of the treatments, on reduction of demineralization or enhancement of remineralization was estimated by the size of ΔS. For both, originally sound and carious slabs, low values mean more effective treatment.

To quantify fluoride concentration on the slabs, the other half of each slab was measured with a digital caliper (± 0.01 mm) and isolated with wax. Alkali-soluble F was determined after KOH extraction (Caslavska et al., 1975) and acid-soluble-F as described by Vale et al. (2011). The results of alkali and acid-soluble F were expressed in $\mu g\ F/cm^2$. Additionally, un-treated slabs and those collected immediately after APF application (Figure 1) were analyzed using the same methodology.

Statistical Analysis

The data for enamel or dentine, sound or carious, were independently analyzed. Volunteers were considered statistical blocks for all variables. Data that did not satisfy assumptions of equality of variances and normal distribution of errors were transformed. The effect of FD concentration (0;1,100;5,000) was evaluated by linear regression analysis. Then, the effect of treatments was checked by ANOVA followed by Tukey test. The analyses were made using SAS system (SAS Institute Inc., version 9.2, Cary, NC, USA) and the significance level was set at 5%.

RESULTS

The dose-response effect for FD concentration (0;1,100;5,000) was significant for most variables (Tables 1 to 4), except to ΔS for carious-dentine (Table 4; p=0.2055). Thus, based on the statistically significant effect of FD concentration on enamel or dentine, de- or remineralization, the effect of the highest concentration (5,000-FD) was compared with the combination effect of professional fluoride application and daily use of 1100-FD (ATF+1,100-FD), considering the aim of the present study.

Regarding enamel, the treatments with 5,000-FD or the combination of APF+1,100-FD did not differ significantly ($p>0.05$), either on the ability to reduce the demineralization (Table 1) or on the property to enhance remineralization (Table 2). For dentine, the combination of APF+1,100-FD was statistically more effective ($p<0.05$) than 5,000-FD either to reduce the area of originally sound dentine (Table 3) or to repair the area of originally carious dentine lesion (Table 4). %SH recovery on dentine slabs are not showed in table 4 because the measurements of SH made after the *in situ* experiment were not reliable. The reactivity of APF-gel and baseline values of alkali and acid-soluble F are shown in the footnote of Tables 1 to 4.

Table 1: Demineralization (%SHL and ΔS) and fluoride concentration (alkali and acid-soluble) in originally **sound enamel** subjected to high caries challenge, and fluoride in biofilm fluid and solids, according to the treatments group (mean±SD;n=15-18)

*Treatments	α %SHL sqr	β ΔS log	γ Alkali-soluble F log (µg F/cm²)	δ Acid-soluble F log (µg F/cm²)	F Biofilm fluid log (µM)	F Biofilm solids log (nM F/g)
PD	55.2±22.9 (n=16)	10,246±5,372 (n=16)	0.28±0.13 (n=16)	2.09±0.73 (n=16)	3.74±2.94 (n=15)	33.6±37.1 (n=16)
1,100-FD	35.4±17.7 (n=18)	5,657±2,739 (n=18)	0.70±0.59 (n=18)	5.12±3.36 (n=18)	6.61±4.48 (n=16)	53.3±40.7 (n=16)
5,000-FD	25.3±16.3 (n=18)	4,460±3,286 (n=18)	1.71±1.73 (n=18)	7.70±4.63 (n=18)	17.4±19.6 (n=18)	254.6±275.7 (n=17)
APF+1,100-FD	[a] 30.5±20.8 (n=17)	[a] 4,429±2,074 (n=17)	[b] 4.27±3.66 (n=17)	[a] 9.94±3.56 (n=17)	[a] 16.9±21.3 (n=16)	[a] 97.1±62.8 (n=15)
p-value from linear regression (0;1,100;5,000 ppm F)	0.0003 sqr	0.0002 log	<0.0001 -0.3	0.0171 sqr	<0.0001 −0.2	<0.0001 -0.3

*PD: placebo fluoride dentifrice; 1,100-FD: fluoride dentifrice (FD), containing 1,100 ppm F; 5,000-FD: FD containing 5,000 ppm F; APF+1,100-FD: combination of acidulated phosphate fluoride application (APF) and 1,100-FD.

α %SHL: percentage of recovery surface harness; β ΔS: area of caries lesions (kg/mm² x µm).

γ Baseline values before and after APF application were 0.11±0.03 and 22.4±25.8 µg F/cm², respectively

δ Baseline values before and after APF application were 1.58±0.81 and 3.11±2.15 µg F/cm², respectively

[a]Superscript letter a indicates that APF+1,100-FD treatment and 5,000-FD did not differ statistically (ANOVA, Tukey, p>0.05)

[b]Superscript letter b indicates that APF+1,100-FD treatment and 5,000-FD differ statistically (ANOVA, Tukey, p<0.05)

sqr,log, -0.1,-0.2, -0.3 Values transformed by: sqr square root; log log10; -0.1 power -0.1; -0.2 power -0.2; -0.3 power -0.3

Table 2: Remineralization (%SHR and ΔS) and fluoride concentration (alkali and acid-soluble) in originally **carious enamel** subjected to low caries challenge, and fluoride in biofilm fluid and solids, according to the treatments group (mean±SD;n=13-18)

*Treatments	α %SHR	β ΔS $^{-0.3}$	γ Alkali-soluble F $^{-0.2}$ (µg F/cm^2)	δ Acid-soluble F ^{log} (µg F/cm^2)	F Biofilm fluid $^{-0.2}$ (µM)	F Biofilm solids ^{log} (nM F/g)
PD	13.6±9.1 (n=16)	9,045±2,706 (n=16)	0.36±0.49 (n=16)	3.94±2.19 (n=16)	4.36±4.39 (n=16)	42.9±75.3 (n=14)
1,100-FD	21.2±12.8 (n=18)	9,197±4,320 (n=18)	0.77±0.71 (n=18)	7.71±4.20 (n=18)	4.86±2.56 (n=13)	196.1±240.3 (n=14)
5,000-FD	30.2±12.7 (n=18)	6,547±1,980 (n=18)	1.80±1.16 (n=18)	12.8±5.78 (n=18)	9.56±9.20 (n=17)	1,052±1,402 (n=16)
APF+1,100-FD	^{a} 29.8±10.7 (n=17)	^{a} 6,255±1,764 (n=17)	^{b} 14.3±12.0 (n=17)¥	^{b} 42.1±10.5 (n=17)	^{a} 37.7±36.8 (n=16)¥	^{a} 648.3±431.9 (n=13)¥
p-value from linear regression (0;1,100;5,000 ppm F)	0.0002	0.0063 $^{-0.3}$	<0.0001 $^{-0.1}$	0.0024 ^{log}	<0.0001 $^{-0.1}$	<0.0001 $^{-0.2}$

*PD: placebo fluoride dentifrice; 1,100-FD: fluoride dentifrice (FD), containing 1,100 ppm F; 5,000-FD: FD containing 5,000 ppm F; APF+1,100-FD: combination of acidulated phosphate fluoride application (APF) and 1,100-FD

α %SHR: percentage of recovery surface harness; β ΔS: area of caries lesions (kg/mm^2 x µm)

γ Baseline values before and after APF application were 0.16±0.04 and 139.4±67 µg F/cm^2, respectively

δ Baseline values before and after APF application were 2.63±1.25 and 12.71±4.19 µg F/cm^2, respectively

[a]Superscript letter a indicates that APF+1,100-FD treatment and 5,000-FD did not differ statistically (ANOVA, Tukey, p>0.05)

[b]Superscript letter b indicates that APF+1,100-FD treatment and 5,000-FD differ statistically (ANOVA, Tukey, p<0.05)

$^{sqr,log,\ -0.1,-0.2,\ -0.3}$ Values transformed by: ^{sqr} square root; ^{log} log10; $^{-0.1}$ power -0.1; $^{-0.2}$ power -0.2; $^{-0.3}$ power -0.3

¥: Outliers removed to combination group, volunteer 10 (KOH-soluble F value 99.7 µg F/cm^2;F fluid value 292 µM F/g.; F solid value 8757 nM F/g)

Table 3: Demineralization (%SHL and ΔS) and fluoride concentration (alkali and acid-soluble) in originally **sound dentine** subjected to high caries challenge, and fluoride in biofilm fluid and solids, according to the treatments group (mean±SD;n=15-18)

*Treatments	α %SHL	β ΔS sqr	γ Alkali-soluble F -0.2 (μg F/cm²)	δ Acid-soluble F sqr (μg F/cm²)	F Biofilm fluid log (μM)	F Biofilm solids log (nM F/g)
PD	50.2±11.4 (n=16)	1,472±673 (n=16)	0.56±0.26 (n=16)	3.63±1.54 (n=16)	3.47±2.30 (n=15)	31.6±36.4 (n=15)
1,100-FD	34.6±11.2 (n=18)	1,034±619 (n=18)	0.97±0.57 (n=18)	10.6±5.03 (n=18)	5.34±3.16 (n=16)	93.1±73.0 (n=15)
5,000-FD	21.3±17.7 (n=18)	677±337 (n=18)	3.18±3.64 (n=18)	17.1±10.8 (n=18)	14.3±15.7 (n=17)	322.9±459.2 (n=16)
APF+1,100-FD	[a] 33.5±13.8 (n=17)	[b] 362±340 (n=17)	[b] 19.4±12.1 (n=17)	[b] 34.1±7.29 (n=17)	[a] 25.1±35.9 (n=17)	[a] 174.5±172.9 (n=16)
p-value from linear regression (0;1,100;5,000 ppm F)	<0.0001	0.0003 sqr	<0.0001 -0.3	<0.0001 sqr	<0.0001 -0.1	<0.0001 -0.2

*PD: placebo fluoride dentifrice; 1,100-FD: fluoride dentifrice (FD), containing 1,100 ppm F; 5,000-FD: FD containing 5,000 ppm F; APF+1,100-FD: combination of acidulated phosphate fluoride application (APF) and 1,100-FD.

α %SHL: percentage of surface hardness loss; β ΔS: area of caries lesions (kg/mm² x μm).

γ Baseline values before and after APF application were 0.13±0.05 and 103.4±48.0 μg F/cm², respectively.

δ Baseline values before and after APF application were 1.34±0.68 and 6.87±1.91 μg F/cm², respectively.

[a]Superscript letter a indicates that APF+1,100-FD treatment and 5,000-FD did not differ statistically (ANOVA, Tukey, p>0.05)

[b]Superscript letter b indicates that APF+1,100-FD treatment and 5,000-FD differ statistically (ANOVA, Tukey, p<0.05)

sqr,log, -0.1,-0.2, -0.3 Values transformed by: sqr square root; log log10; -0.1 power -0.1; -0.2 power -0.2; -0.3 power -0.3

Table 4: Remineralization (%SHR and ΔS) and fluoride concentration (alkali and acid-soluble) in originally **carious dentine** subjected to low caries challenge, and fluoride in biofilm fluid and solids, according to the treatments group (mean±SD;n=14-18)

*Treatments	[α] %SHR	[β] ΔS	[γ] Alkali-soluble F $^{-0.2}$ (μg F/cm^2)	[δ] Acid-soluble F ^{log} (μg F/cm^2)	F Biofilm fluid $^{-0.3}$ (μM)	F Biofilm solids $^{-0.1}$ (nM F/g)
PD	nd	1,130±412 (n=16)	0.41±0.16 (n=16)	4.36±1.03 (n=16)	3.32±2.27 (n=16)	61.1±98.6 (n=14)
1,100-FD	nd	1,294±503 (n=18)	0.70±0.33 (n=18)	12.0±5.64 (n=18)	4.03±2.17 (n=16)	261.4±281.0 (n=16)
5,000-FD	nd	1,033±472 (n=18)	1.51±0.75 (n=18)	22.8±13.2 (n=18)	7.47±6.51 (n=16)	734.8±834.1 (n=16)
APF+1,100-FD	nd	[b] 360±272 (n=17)	[b] 46.5±37.7 (n=16)¥	[b] 24.9±9.83 (n=17)	[a] 106.9±151.1 (n=17)	[a] 477.8±336.8 (n=14)
p-value from linear regression (0;1,100;5,000 ppm F)	nd	0.2055 ^{sqr}	<0.0001 ^{log}	<0.0001 ^{sqr}	<0.0001 ^{log}	<0.0001 ^{log}

*PD: placebo fluoride dentifrice; 1,100-FD: fluoride dentifrice (FD), containing 1,100 ppm F; 5,000-FD: FD containing 5,000 ppm F; APF+1,100-FD: combination of acidulated phosphate fluoride application (APF) and 1,100-FD.

nd: %SHR was not calculated

[α] %SHR: percentage of recovery surface harness; [β] ΔS: area of caries lesions (kg/mm^2 x μm).

[γ] Baseline values before and after APF application were 0.23±0.10 and 239.5±135.3 μg F/cm^2., respectively.

[δ] Baseline values before and after APF application were 2.94±0.82 and 10.91±3.6 μg F/cm^2., respectively.

[a]Superscript letter a indicates that APF+1,100-FD treatment and 5,000-FD did not differ statistically (ANOVA, Tukey, p>0.05)

[b]Superscript letter b indicates that APF+1,100-FD treatment and 5,000-FD differ statistically (ANOVA, Tukey, p<0.05)

$^{sqr,log,\ -0.1,-0.2,\ -0.3}$ Values transformed by: ^{sqr} square root; ^{log} log10; $^{-0.1}$ power -0.1; $^{-0.2}$ power -0.2; $^{-0.3}$ power -0.3

¥: Outlier removed to combination group, volunteer 10, value = 163.9 μg F/cm^2.

DISCUSSION

Since gingival recession exposes enamel and root dentine to the same environmental condition, it is reasonable that caries and its control in these dental substrates should be studied simultaneously. Also, given that root-dentine is more caries-susceptible than enamel it is rational to think that additional fluoride (F) treatments may be more relevant to improve F effect on root caries control than on enamel. Nevertheless, the mechanism of action of fluoride on enamel and dentine caries process is basically the same. Fluoride interferes with the caries process, reducing demineralization and enhancing remineralization either of enamel or dentine. Therefore, in the present *in situ* study, we simulated conditions to evaluate simultaneously the effect of fluoride treatments on enamel and dentine de- and remineralization. The first condition tested the effect of fluoride on reduction of demineralization that occurs in sound teeth ("preventive effect") when subjected to a high cariogenic challenge (de->re-). At same time, it was simulated a condition of low cariogenic challenge (re->de-) to evaluate the effect of fluoride on enhancement of remineralization, when caries lesions already present could be repaired ("therapeutic effect"). We used a widely tested *in situ* model (Tenuta and Cury, 2013) to test the hypotheses raised, solving ethical issues to conduct clinical studies simulating caries and using placebo fluoride dentifrice. Furthermore, clinical trials based on net DMF changes are not able to differentiate fluoride preventive from therapeutic effect (Baelum et al., 2003).

The significant dose-response effect for FD-concentration (0;1,100;5,000) on enamel de-remineralization extends the conclusion based on evidence that the "caries preventive effect" of FD is concentration dependent (Marinho et al., 2003; Walsh et al., 2010), considering that effect dose-response was also significant for the "therapeutic" effect of FD repairing caries lesions (Tables 1 and 2). The FD dose-response effect found for either enamel demineralization (Tables 1) or remineralization (Table 2), the main outcomes of our study, were consistent with the significant dose-response effects also found for all variables evaluated. The secondary variables (fluoride in enamel (acid and alkali-soluble) and fluoride

in biofilm (fluid and solids), were chosen to give support to the data of mineral lost and gain and also showed dose-response to FD concentration.

Concerning to dentine, it was also found effect dose-response of FD concentration on reduction of demineralization (Table 3), which showed coherence with data of F-concentration in biofilm and fluoride in dentine. These data suggest that the effect of FD on reduction of caries initiation in dentine (Table 3) is similar than in enamel (Table 1), but the effect on dentine remineralization (Table 4) was lower than that found on enamel (Table 2). A "therapeutic effect" of fluoride (remineralization) was found for enamel, even in the presence of biofilm accumulation and sugar exposure 3x/day. However, the same was not observed for dentine, suggesting that meticulous toothbrushing with FD should be more relevant to repair caries on dentine than on enamel (Nyvad, 2008). Additionally, these data suggest that more fluoride would be necessary to control root dentine caries than enamel, what could be achieved by the combination of regular daily toothbrushing with FD with professional fluoride application, or the use of 5,000-FD. Considering that dentine is more reactive than enamel, it would be expected that the combination APF+1,100-FD would be as effective or better as 5,000-FD use.

Indeed, our results showed that for enamel the use of APF associated with 1,100-FD did not have better effect than 5,000-FD, either on demineralization or remineralization (Table 1 and 2). These data are coherent with the no difference between APF+1,100-FD and 5,000-FD for fluoride concentration in biofilm, although biofilm has been collected 10 h after the toothbrushing. For dentine, the combination of APF+1,100-FD was more effective than 5,000-FD either on reduction of the area of caries lesion (ΔS, Table 3) or on to repair the area of caries lesion (ΔS, Table 4). The findings are supported by the relative greater alkali-soluble fluoride concentration found in dentine than enamel for APF-1,100-FD compared with 5,000-FD. Theoretically the fluoride released from these reservoirs may have been used to interfere with the caries process (Tenuta et al., 2009) and to increase the concentration of firmly bound fluoride (acid-soluble) in slabs. The finding about the comparison between 5,000-FD and APF+1,100-FD is relevant because 5,000-FD has been recommended to root caries control

rather than 1,100-FD (Petersson, 2013; Wierichs and Meyer-Lueckel, 2014), but high-F concentration dentifrices are not over-the-counter products for oral hygiene. As 5,000-FD is a prescription product, the combination of 1,100-FD, an over-the-counter dentifrice, with APF is an alternative that in addition seems more effective than the use of 5,000-FD for root caries control.

Our findings showing coherence between concentration of FD and the dose-response effects observed either for fluoride in biofilm fluid and fluoride in dental substrates are supported by the mechanisms how FD control caries (Tenuta and Cury, 2013). However, fluoride incorporated on dental substrates should be considered a consequence of the dynamic of caries process (de-remineralization) rather than the reason for the effect on caries inhibition or reversal. In presence of fluoride in biofilm fluid dental tissues are enriched with fluoride during the caries process. However, although it is considered that fluorapatite is deposited during the caries process (Larsen, 1973), our data showed that loosely bound fluoride (alkali-soluble) were found according to FD concentration. Also, the formation of loosely bound F on dental surfaces cannot be attributed to the chemical reaction that could happen every time that FD was used, because slabs were covered by biofilm. On the other hand, it has been shown experimentally that the concentration of loosely bound F formed on clean dental surfaces is very low to have an effect of reduction of demineralization (Tenuta et al., 2009; Tenuta and Cury, 2013). Nevertheless, the findings are relevant because loosely bound fluoride formed during the caries process may work as reservoirs of fluoride, releasing fluoride to biofilm between the intervals of toothbrushing to interfere with the caries process.

APF application on slabs of group APF+1,100-FD formed high concentration of alkali-soluble F on enamel and dentine, but this concentration decreased 80 to 90% during 14 days into the mouth under cariogenic challenge. That suggest that this reservoir of fluoride was dissolved by saliva or depressed by the caries process (Ogaard, 2001). Therefore, the daily use of FD is important to maintain F in saliva and in biofilm fluid after brushing, and also should be relevant to maintain the reservoirs of F on dental surfaces.

In spite of the fact that F on the biofilm showed a high standard deviation, that difficult to found differences among groups, the fluoride in the fluid and solid portion showed dose-response by FD-concentration (Tables 1 to 4). Outstandingly, the F in fluid of biofilm after 5,000-FD use was explored for the first time in the present study, showing be able to maintain a relative important concentration of F in the biofilm, even evaluated after 10 h of the last toothbrushing. Furthermore, reservoirs of fluoride were also found in biofilm solids according FD concentration, which could be released to interfere with caries progression.

In conclusion, the findings suggest that either the inhibition of demineralization or enhancement of remineralization of either enamel or root-dentine are FD-concentration dependent and, that the combination of APF+1,100-FD would be relevant only to control root-dentine caries.

ACKNOWLEDGMENTS

We thank to the volunteers for their valuable participation. This study is based on the first author's Doctorate Thesis (Doctorate Program in Dentistry, Cariology Area, Faculty of Dentistry of Piracicaba, State University of Campinas, UNICAMP, Brazil). The first author received scholarship during this study by Government of Chile, National Commission for Scientific and Technological Research (CONICYT) Res.1166/2011. This study was partially supported by Funcamp (Conv. 4252). There are no conflicts of interest with respect to the authorship and/or publication of this article. J.A. Cury occasionally acted as a consultant to Colgate/Palmolive in Brazil and to Brazilian Ministry of Health during the time this study was conducted. Partials results were presented in 61th ORCA Congress, 2014, Greifswald, Germany.

REFERENCES

Baelum V, Machiulskiene V, Nyvad B, Richards A, Vaeth M (2003). Application of survival analysis to carious lesion transitions in intervention trials. *Community Dent Oral Epidemiol* 31(4)*:*252-260.

Baysan A, Lynch E, Ellwood R, Davies R, Petersson L, Borsboom P (2001). Reversal of primary root caries using dentifrices containing 5,000 and 1,100 ppm fluoride. *Caries Res* 35(1)*:*41-46.

Botelho JN, Del Bel Cury AA, Silva WJ, Tenuta LM, Cury JA (2014). The effect of fluoride toothpaste on root dentine demineralization progression: a pilot study. *Braz Oral Res* 28(spe)*:*1-5.

Calvo AF, Tabchoury CP, Del Bel Cury AA, Tenuta LM, da Silva WJ, Cury JA (2012). Effect of acidulated phosphate fluoride gel application time on enamel demineralization of deciduous and permanent teeth. *Caries Res* 46(1)*:*31-37.

Caslavska V, Moreno EC, Brudevold F (1975). Determination of the calcium fluoride formed from in vitro exposure of human enamel to fluoride solutions. *Arch Oral Biol* 20(5-6)*:*333-339.

Ccahuana-Vasquez RA, Tabchoury CP, Tenuta LM, Del Bel Cury AA, Vale GC, Cury JA (2007). Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride. *Caries Res* 41(1)*:*9-15.

Cury JA, Tenuta LM (2014). Evidence-based recommendation on toothpaste use. *Braz Oral Res* 28 Spec no. 1(1-7).

Cury JA, do Amaral RC, Tenuta LM, Del Bel Cury AA, Tabchoury CP (2010). Low-fluoride toothpaste and deciduous enamel demineralization under biofilm accumulation and sucrose exposure. *Eur J Oral Sci* 118(4)*:*370-375.

Cury JA, Tenuta LM (2008). How to maintain a cariostatic fluoride concentration in the oral environment. *Adv Dent Res* 20(1)*:*13-16.

Cury JA, Rebelo MA, Del Bel Cury AA, Derbyshire MT, Tabchoury CP (2000). Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. *Caries Res* 34(6)*:*491-497.

Ekstrand KR, Poulsen JE, Hede B, Twetman S, Qvist V, Ellwood RP (2013). A randomized clinical trial of the anti-caries efficacy of 5,000 compared to 1,450 ppm fluoridated toothpaste on root caries lesions in elderly disabled nursing home residents. *Caries Res* 47(5)*:*391-398.

Fernández CE, Tenuta LMA, Cury JA (2015). Wash-out period for crossover design experiments using high fluoride concentration dentifrice *Rev Clin Periodoncia Implantol Rehabil Oral*. Ref.PIRO-D-14-00009 [Accepted].

Hara AT, Queiroz CS, Paes Leme AF, Serra MC, Cury JA (2003). Caries progression and inhibition in human and bovine root dentine in situ. *Caries Res* 37(5)*:*339-344.

Heijnsbroek M, Paraskevas S, Van der Weijden GA (2007). Fluoride interventions for root caries: a review. *Oral Health Prev Dent* 5(2)*:*145-152.

Kidd E, Fejerskov O (2013). Changing concepts in cariology: forty years on. *Dental update* 40(4)*:*277-278, 280-272, 285-276.

Larsen MJ (1973). Dissolution of enamel. *Scand J Dent Res* 81(7)*:*518-522.

Marinho VC, Higgins JP, Sheiham A, Logan S (2003). Fluoride toothpastes for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* 1)*:*CD002278.

Moreno EC, Zahradnik RT (1974). Chemistry of enamel subsurface demineralization in vitro. *J Dent Res* 53(2)*:*226-235.

Nyvad B (2008). The role of oral hygiene In: Dental caries The disease and its clinical management. O Fejerskov and E Kidd editors. Oxford: Blackwell Munksgaard, pp. 257-264.

Nyvad B, Fejerskov O (1982). Root surface caries: clinical, histopathological and microbiological features and clinical implications. *Int Dent J* 32(4)*:*311-326.

Ogaard B (2001). CaF(2) formation: cariostatic properties and factors of enhancing the effect. *Caries Res* 35 Suppl 1(40-44.

Paes Leme AF, Dalcico R, Tabchoury CP, Del Bel Cury AA, Rosalen PL, Cury JA (2004). In situ effect of frequent sucrose exposure on enamel demineralization and on plaque composition after APF application and F dentifrice use. *J Dent Res* 83(1)*:*71-75.

Paes Leme AF, Tabchoury CP, Zero DT, Cury JA (2003). Effect of fluoridated dentifrice and acidulated phosphate fluoride application on early artificial carious lesions. *Am J Dent* 16(2)*:*91-95.

Petersson LG (2013). The role of fluoride in the preventive management of dentin hypersensitivity and root caries. *Clin Oral Investig* 17 Suppl 1(S63-71.

Queiroz CS, Hara AT, Paes Leme AF, Cury JA (2008). pH-cycling models to evaluate the effect of low fluoride dentifrice on enamel de- and remineralization. *Braz Dent J* 19(1)*:*21-27.

Srinivasan M, Schimmel M, Riesen M, Ilgner A, Wicht MJ, Warncke M *et al.* (2014). High-fluoride toothpaste: a multicenter randomized controlled trial in adults. *Community Dent Oral Epidemiol* 42(4)*:*333-340.

ten Cate JM (2013). Contemporary perspective on the use of fluoride products in caries prevention. *Br Dent J* 214(4)*:*161-167.

ten Cate JM, Damen JJ, Buijs MJ (1998). Inhibition of dentin demineralization by fluoride in vitro. *Caries Res* 32(2)*:*141-147.

Tenuta LM, Cury JA (2013). Laboratory and human studies to estimate anticaries efficacy of fluoride toothpastes. *Monogr Oral Sci* 23(108-124).

Tenuta LM, Zamataro CB, Del Bel Cury AA, Tabchoury CP, Cury JA (2009). Mechanism of fluoride dentifrice effect on enamel demineralization. *Caries Res* 43(4)*:*278-285.

Tenuta LM, Del Bel Cury AA, Bortolin MC, Vogel GL, Cury JA (2006). Ca, Pi, and F in the fluid of biofilm formed under sucrose. *J Dent Res* 85(9)*:*834-838.

Vale GC, Tabchoury CP, Del Bel Cury AA, Tenuta LM, ten Cate JM, Cury JA (2011). APF and dentifrice effect on root dentin demineralization and biofilm. *J Dent Res* 90(1)*:*77-81.

Vogel GL, Mao Y, Carey CM, Chow LC (1997). Increased overnight fluoride concentrations in saliva, plaque, and plaque fluid after a novel two-solution rinse. *J Dent Res* 76(3)*:*761-767.

Walls AW, Meurman JH (2012). Approaches to caries prevention and therapy in the elderly. *Adv Dent Res* 24(2)*:*36-40.

Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X (2010). Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* 1)*:*CD007868.

White DJ (1987). Reactivity of fluoride dentifrices with artificial caries. I. Effects on early lesions: F uptake, surface hardening and remineralization. *Caries Res* 21(2)*:*126-140.

Wierichs RJ, Meyer-Lueckel H (2014). Systematic Review on Noninvasive Treatment of Root Caries Lesions. *J Dent Res*.

CONSIDERAÇÕES*

Esta tese teve como objetivo principal estudar o efeito de fluoreto (F) tanto no esmalte como na dentina radicular, considerando que em pacientes com recessão gengival ambos substratos dentais estão expostos às mesmas condições ambientais. Assim, o conhecimento gerado nesta pesquisa é relevante para o entendimento do controle da cárie coronária (esmalte) o que é importante não somente para crianças e adultos, mas também para o controle da cárie radicular (dentina radicular), extremamente importante para adultos e idosos.

Em geral, observamos que o F fornecido por uma solução fluoretada (capítulo 1), simulando o uso de dentifrício fluoretado (DF), ou o uso de DF e sua combinação com aplicação profissional de fluoreto (APF) (capítulo 2) desempenham um papel evidente no controle da cárie, reduzindo a desmineralização e ativando a remineralização, tanto do esmalte como da dentina. Nossos resultados *in vitro e in situ* convergiram para concluir que em dentina são necessárias maiores concentrações de fluoreto ou combinação de meios de uso de F para o controle da cárie.

Os dados do modelo *in vitro* validado mostraram que existem notáveis diferenças do efeito que o F tem sobre esmalte e dentina radicular. Como foi descrito no capítulo 1, com base na %PDS, seria necessária uma concentração de F 3 vezes maior para dentina para ter a mesma porcentagem de efeito de redução de desmineralização que a observada para o esmalte. Isto é justificável, pois a dentina radicular é mais vulnerável à cárie dentária que o esmalte dental devido à sua composição (Nyvad e Fejerskov, 1982), e assim progride mais rapidamente que o esmalte (Ogaard et al., 1988). Além disso, a informação proporcionada pelo estudo *in vitro* mostra claramente que o efeito do F foi físico-químico, reduzindo a desmineralização dos substratos, sem efeito antimicrobiano, uma vez que não houve efeitos dos tratamentos na acidogenicidade do biofilme. Assim, o Capítulo 1 além de mostrar efeito dose-resposta ao uso de F no modelo de biofilme, deixou evidente que maior concentração de F ou possivelmente a combinação de meios

* *Tópico opcional para apresentação de Tese segundo a deliberação CCPG-Nº 228/2013 que regula as normas sobre o formato e a impressão de dissertação e/ou tese da Universidade Estadual de Campinas.*

seria necessária para o controle de cárie na dentina radicular. Uma primeira e ideal opção seria o uso diário de DF de alta concentração (DF-5000) que tem mostrado melhor efeito anticárie na dentina que DF de concentração convencional (DF-1100) (Baysan et al., 2001; Ekstrand et al., 2013; Srinivasan et al., 2014). Porém, para a sua aquisição é necessária prescrição. Uma alternativa a DF-5000 seria DF-1100, o qual é de livre venda, combinado com APF, entretanto a comparação destes dois meios de uso de F (DF-5000 ou combinação APF+DF-1100) não tem sido estudada previamente.

No capítulo 1 foi estudado somente o efeito do fluoreto na redução da desmineralização, e não na remineralização. Além disso, seria esperado que o efeito de DF fosse concentração dependente para esmalte e dentina na des- e também na remineralização, e hipoteticamente o efeito da combinação (APF+DF-1100) poderia ser igual ou superior ao uso de DF-5000 na dentina. Assim, com base em nossos resultados *in vitro* (capítulo 1) a próxima etapa do trabalho foi avaliar *in situ* o efeito dose-resposta de DF e o efeito comparativo de dentifrício de alta concentração de F (DF-5000 ppm) com a combinação da aplicação profissional de fluoreto (FFA: flúor fosfato acidulado) mais o uso diário de dentifrício de concentração convencional (DF-1100 ppm), onde adicionalmente ao estudo de efeito na redução da desmineralização, estudamos simultaneamente a ativação da remineralização do esmalte e dentina radicular.

Para essa comparação utilizamos um modelo de estudo *in situ* (capítulo 2), com biofilme complexo formado naturalmente na cavidade bucal dos sujeitos de pesquisa. A grande vantagem desse modelo *in situ* é avaliar o processo de cárie em condições controladas e mais próximas à realidade. Para isso, o próprio biofilme do sujeito de pesquisa é acumulado nos blocos tendo também a influência dos fatores salivares, mas tendo a opção de recuperar as amostras e utilizar como desfechos a integração de diversas técnicas analíticas laboratoriais de alta sensibilidade e validade científica (Zero, 1995). Em condições *in vivo* o acúmulo de biofilme é complicado e também não podem ser utilizados métodos precisos para avaliação quantitativa da perda mineral por serem destrutivos. Também, por razões éticas (simulação de cárie e uso de dentifrício placebo) este estudo não poderia ser conduzido *in vivo*. Além do que a alta variabilidade entre indivíduos requereria a inclusão de muitos sujeitos em um ensaio clínico, aumentando consideravelmente seu

custo e dificuldade de execução. Adicionalmente, o uso deste modelo nos permite separar e diferenciar o efeito que o fluoreto tem para inibir a desmineralização ou potencializar a remineralização. Isso seria impossível de estudar *in vivo*, embora seja clinicamente conhecido que lesões de cárie possam progredir, ser paralisadas ou revertidas, mas o diagnóstico e estudo é dificultado. Tudo isso favoreceu optarmos pelo uso de modelo *in situ* para o desenvolvimento desta tese, além disso, trabalhos prévios do laboratório de Bioquímica Oral da FOP/UNICAMP, utilizando essa metodologia, têm mostrado resultados em concordância com as melhores evidências sobre o uso de fluoretos no controle da cárie, dando credibilidade e força a essa metodologia.

Em relação aos resultados do estudo *in situ* (Capítulo 2), foi observado mediante análise de regressão um efeito claro e significativo ($p<0,05$) da concentração (0; 1100; 5000 ppm F) para todas as variáveis respostas (exceto ΔS para dentina cariada), o que valida o nosso modelo e ratifica a presença de dose-resposta. Considerando o efeito significativo da concentração, o grupo mais efetivo foi DF-5000 ppm, logo o passo seguinte foi comparar esse grupo com a combinação de meios (APF+DF-1100). Para esmalte não foram observadas diferenças do efeito dos tratamentos com adicional de F (DF-5000 ppm ou APF+DF-1100) tanto na des- (Cap. 2; Tabela 1; pág. 32) nem na remineralização (Cap. 2; Tabela 2; pág. 33). A combinação APF+DF-1100 resultou ser mais efetiva na dentina, quer seja para redução da desmineralização da dentina hígida como para ativação da remineralização da dentina cariada. Esse melhor efeito seria explicado pela alta concentração de depósitos de F solúvel em álcali ("CaF_2"), que estaria disponível para aumentar a concentração de F no fluido do biofilme. Foi observado que os valores médios de "CaF_2" avaliados após a exposição *in situ* foram 6 e 30 vezes maiores para APF+DF-1100 do que para DF-5000, na dentina-hígida e dentina-cariada, respetivamente, (Cap. 2; Tabela 3 e 4; pág. 34 e 35). Esse fluoreto poderia ser utilizado para reduzir a desmineralização e ativar a remineralização. Estes resultados para dentina estão em concordância com estudos *in situ* prévios, onde a combinação de dentifrício fluoretado com métodos de aplicação profissional (APF) pode representar um benefício adicional e sinérgico para o controle de cárie na dentina radicular (Vale et al., 2011).

No trabalho *in situ*, avaliamos o efeito do F na redução da desmineralização e na ativação da remineralização num mesmo experimento, usando substratos hígidos ou cariados respetivamente para avaliar a importância relativa do F nesses processos. Deste modo, no nosso delineamento experimental foram simuladas duas condições clínicas, ambas com acúmulo de biofilme, mas diferindo na frequência de exposição à sacarose. Na primeira situação substratos hígidos foram expostos à alta frequência de uso de sacarose (8x/dia), condição de alto desafio cariogênico de Des- > Re-, quando foi avaliado o efeito do F na redução da desmineralização (Des > Re) ("efeito preventivo"). Na outra situação substratos desmineralizados foram expostos à baixa frequência de sacarose (3x/dia), simulando condição clínica de baixo desafio cariogênico (Re- > Des-) para estudar a reversão de lesões de cárie pré-existentes ("efeito terapêutico"). Deve ser enfatizado, que cárie é um processo dinâmico de flutuações de episódios de Des- e Re-, os quais ocorrem no meio bucal, porém em cada uma das condições simuladas nesse estudo, o tempo de ocorrência de um processo predominou sobre o outro.

Mesmo sob um desafio cariogênico de exposição à sacarose 8x/dia, o F foi capaz de reduzir a progressão das lesões de cárie tanto no esmalte como na dentina, apesar dessa ser considerada um mineral mais solúvel (Hoppenbrouwers et al., 1986). Tendo altas concentrações de F disponibilizadas mediante uma fonte de uso diário, como foi o DF-5000 ppm utilizado 2x/dia, ou com o uso de APF (flúor-fosfato acidulado (FFA) uma aplicação no início do experimentado) associado com uso de DF-1100 ppm utilizado também 2x dia, permitiu disponibilizar uma alta concentração de F no fluido do biofilme, além do F que possa ser mantido na saliva pelo uso de DF. O F presente no microambiente ao redor do substrato poderia ser utilizado considerando o mecanismo físico-químico pelo qual o F atua, assim, quando o pH estiver abaixo do valor crítico vai reduzir a desmineralização pela reposição parcial do mineral perdido e quando o pH for acima do crítico o F presente na saliva vai ter o potencial para remineralizar, especialmente nos blocos com lesões prévias (Cury e Tenuta, 2008, 2009).

Tanto no estudo *in vitro* como no *in situ* foi avaliado apenas o efeito do F do dentifrício porque o efeito da escovação não foi simulado. Nos dois modelos os biofilmes não foram submetidos à remoção/desorganização mecânica, favorecendo o acúmulo para simular uma

condição altamente cariogênica. No estudo *in situ* essa condição altamente cariogênica permite que num curto período de tempo (14 dias) seja possível estudar o efeito dos tratamentos o qual ocorreria *in vivo*, porém depois de um longo período de tempo. Se o biofilme fosse removido/desorganizado mediante escovação o efeito do F seria potencializado, pois a deposição de minerais que ocorre na interface dente-biofilme seria melhorada na exposição direta de F que provem da escovação regular com DF (Nyvad e Fejerskov, 1986; Nyvad, 2008). Assim, quando o biofilme for removido pela escovação o F presente na saliva poderá ativar a remineralização dessas superfícies dentais limpas pela escovação (Tenuta e Cury, 2013). Entretanto, considerando que o principal efeito do F é atuar nos lugares onde o biofilme dental não é totalmente removido (Tenuta et al., 2009) os nossos resultados são amplamente validos e úteis. Simulamos superfícies não perfeitamente limpas pela escovação, onde fluoreto se difunde e é retido no fluido do biofilme para ter efeito no controle da cárie (Cury e Tenuta, 2014).

A escovação com dentifrício fluoretado tem como objetivo aumentar a concentração de F na boca (saliva e biofilme) momentaneamente até o *clearance* salivar diluir o F presente. O F proveniente DF pode ficar retido em residuais de biofilme não perfeitamente removidos pela escovação ou reagir com as superfícies dentais limpas pela escovação formando F solúvel em álcali ("CaF_2") (Tenuta e Cury, 2013). Para dentífricos, a formação destes produtos não seria relevante, pois foi experimentalmente mostrado que a concentração de F fracamente ligado em superfícies limpas é muito baixa para ter efeito na redução da desmineralização (Tenuta et al., 2009; Tenuta e Cury, 2013). No entanto, a formação destes produtos é o principal mecanismo de ação de produtos de APF. Nos grupos utilizando dentífricos fluoretados (DF-1100 e DF-5000 ppm) a presença de F solúvel em álcali não poderia ser atribuída a reação química que ocorreria cada vez que DF entra em contato com as superfícies dentárias, pois os blocos estiveram cobertos por biofilme dental. Assim, esses dados deixam evidente que possivelmente essa formação não ocorreu somente devido a uma reação química entre F e a superfície dentária nas primeiras horas do experimento quando as superfícies estiveram limpas. É possível pensar também que esse mineral seria formado como consequência do processo de cárie. No grupo de DF-5000 ppm as quantidades achadas foram duas a três vezes maiores do que DF-1100 ppm, então além do aumento momentâneo durante a escovação, DF-5000 ppm aporta F de maneira que ele não

somente aumenta a concentração de F no biofilme, mas também consegue disponibilizar F para ser precipita e formar depósitos na superfície.

Hipoteticamente estes reservatórios de F formados nas superfícies dentárias irão liberar fluoreto para o fluido do biofilme (Tenuta et al., 2008) frente às quedas de pH (Ogard et al., 1994), induzidas pela exposição a açúcares fermentáveis, reduzindo a desmineralização e aumentando a remineralização (Tenuta e Cury, 2010). Assim, lesões de cárie irão progredir ou se reverter dependendo do equilíbrio des-re a que os dentes serão submetidos diariamente na cavidade bucal (Kidd e Fejerskov, 2013). Assim sendo, nós explicamos o melhor efeito da combinação APF+DF-1100 frente ao DF-5000 ppm na dentina radicular, baseados na dissolução destes reservatórios para remineralizar a superfície, os quais foram significativamente maiores que para DF-5000 ppm. No caso do esmalte, ele não se viu beneficiado por maiores concentrações de F solúvel em álcali nas superfícies dentárias. Uma possível explicação considera que o desenvolvimento de lesões de cárie é semelhante em esmalte e dentina, mas acorrerá com base no equilíbrio de dissolução de um mineral (ten Cate et al., 2008). Assim, para a precipitação mineral é necessária a presença de outros minerais além do F (p.e. cálcio e fosfato) e em um grau de saturação para favorecer a precipitação de mineral na forma de FAp (Tenuta e Cury, 2004). Então, teoricamente como o esmalte é menos reativo que a dentina, consequentemente tem menos capacidade para precipitar mineral. Entretanto, no estudo *in vitro* o F teve marcadamente um maior efeito no esmalte que na dentina, situação que seria explicada considerando que estivemos frente a fases iniciais de desmineralização. Já no *in situ*, as lesões tiveram maior progressão e essas diferenças entre esmalte e dentina diminuíram (p.e. 55,2 e 50,2 para %SHL em esmalte e dentina respectivamente (Cap.2; Tabela 1; pág. 32 e Tabela 2; pág. 33).

Por outro lado, o flúor fosfato acidulado formou no primeiro dia uma alta concentração de F solúvel em álcali ("CaF_2"), e essa concentração foi altamente efetiva e mantida até duas semanas depois (aprox. 10-20%). Mas, levando em consideração que FFA é um meio de APF que usualmente é recomendado semestralmente, estamos frente a um efeito marcado dos primeiros dias de liberação, o qual poderia ser reduzido se o período experimental fosse maior. Hipoteticamente então, num estudo de longa duração (idealmente *in vivo*) o efeito de ambos

poderia ser o mesmo ou superior para DF-5000 ppm se usado corretamente, considerando que é uma medida de auto-aplicação recomendada para uso diário.

O fluido do biofilme é considerado um retrato momentâneo do processo dinâmico de perda e ganho mineral que ocorre na interface dente/biofilme. No presente estudo as análises de F no fluido foram realizadas aproximadamente 10 horas após a última escovação, tempo no qual é possível atingir uma situação de equilíbrio. Entretanto, nós observamos que DF teve a capacidade de aumentar as concentrações de F no biofilme e ser sensível para diferenciar os tratamentos de DF nas diferentes concentrações utilizadas, inclusive sendo esta análise 10 h depois da última escovação. No entanto, o grupo APF-1100 não foi diferente do grupo DF-5000. Uma plausível explicação seria que para o grupo da combinação, além de ter sido dissolvida uma grande porcentagem de mineral tipo "CaF_2" em 14 dias (80 a 90%), a área de reação foi pequena (0,16 cm^2), portanto no final do período experimental (depois de 14 dias), esses reservatórios estariam liberando somente pequenas concentrações para o fluido do biofilme. Pelo outro lado, o DF-5000 conseguiria manter altos valores de F no fluido do biofilme depois de 10 h da última escovação sendo ele utilizado 2x/dia. Além disso, foi observada uma alta variabilidade dos dados do biofilme o que dificulta detectar diferenças estatisticamente significativas entre os tratamentos, tendo em vista o tamanho de amostra usado.

Foi observado também que os valores do fluido foram muito maiores que os valores de F achados na porção sólida do biofilme. Essa maior concentração pode vir das exposições diárias a F durante as escovações e ser retido no fluido, mas também pode surgir desde a dissolução de reservatórios de F das superfícies dentárias ou do F ligado ao biofilme frente às quedas de pH.

A análise da porção sólida do biofilme mostrou que reservatórios podem ser formados inclusive pelo uso de DF-1100 ppm, mas a medida que aumentamos a concentração do F no dentifrício (para 5000 ppm) formamos mais depósitos fortemente ligados ao biofilme. O grupo da combinação não apresentou maiores depósitos de F do que DF-5000 ppm, concordando como o efeito similar em cárie dentária no esmalte na de- e na remineralização.

Considerações metodológicas

O estudo *in vitro* (Capítulo 1) teve como inovação a montagem simultânea de esmalte e dentina radicular num mesmo poço da placa, sendo submetidos a condições idênticas durante todo o experimento. Isto possibilitou o estudo simultâneo do processo de cárie em ambos os substratos. O modelo original de Ccahuana-Vasquez e Cury, 2010 desenvolvido somente para esmalte no início, também trabalhou com esmalte e dentina radicular separadamente em diferentes poços e com diferentes tempos de formação de biofilme (Giacaman et al., 2012; Munoz-Sandoval et al., 2012). No nosso estudo *in vitro*, depois de vários testes, foi encontrado que 96 h conseguiu ser um tempo suficiente para que os dois substratos mostrassem diferenças entre os grupos de tratamento (efeito dose-resposta). Além disso, o tempo de duração do experimento permitiu estudar os estágios iniciais de cárie dentária nos quais ocorre somente o efeito na troca de minerais, sem chegar à desintegração da matriz proteica da dentina radicular, o que suporta o uso de dureza de superfície como principal desfecho. No experimento *in situ* (apêndice 5, pág. 64) 4 blocos hígidos (2 de esmalte e 2 de dentina) foram montados num lado do aparelho, enquanto que outros 4 blocos, cariados, foram postos do outro lado. Para metade dos sujeitos de pesquisa, os blocos hígidos foram montados do lado direito e para a outra metade dos sujeitos do lado esquerdo. Além disso, a sequência da posição anterior para posterior dos 4 blocos de cada lado também foi prefixada. Para metade dos sujeitos começou com esmalte, enquanto a outra metade começou com dentina. Estas posições foram fixadas para cada sujeito durante os quatro fases do estudo. Desse modo tentamos fazer com que as amostras estivessem sob a mesma condição ambiental, sem introduzir um viés pela posição. Nestes dados *in situ* não fizemos uma comparação direta entre esmalte e dentina, assim, os substratos foram analisados independentemente em cada condição (hígida ou cariada).

Por outro lado, a utilização de dentina radicular como representativa do tecido radicular é consenso, pois apesar da dentina radicular não exposta estar coberta por uma camada de cemento, a cárie radicular normalmente se desenvolve na dentina (Berry et al., 2004). O cemento na região cervical tem uma espessura entre 20–50 µm (Jang et al., 2014) e quando a superfície radicular está exposta, êle está parcial ou totalmente removido (Fejerskov et al., 2008). Essa remoção pode ser mecânica durante procedimentos de raspagem radicular ou

escovação dental, ou remoção química por dissolução ácida, portanto a dentina radicular fica exposta à formação de biofilme e ao processo de cárie (ten Cate et al., 2008).

A dureza de superfície foi usada nos dois trabalhos (capítulos 1 e 2) como indicador de desmineralização ou remineralização, pois ela tem mostrado uma alta correlação com a perda mineral avaliada com micrografia transversal em esmalte (White, 1987) e também na dentina (Vale et al., 2011). Assim está técnica tem sido amplamente utilizada e com reconhecida sensibilidade para estudo de cárie (Zero, 1995). No entanto, na dentina-cariada tivemos uma limitação metodológica, onde não foi possível quantificar a dureza de superfície devido a dificuldades de delimitar as edentações. Assim, na dentina previamente desmineralizada a dureza em corte se tornou fundamental. Porém, não foi observado efeito da concentração dos grupos tratados com dentifrício fluoretado para ΔS (análise de regressão, Cap. 2; Tabela 4; pág. 35; p=0,2055). A não diferença estatística entre os grupos tratados com DF de diferentes concentrações (0; 1100; 5000) poderia ser explicada pela condição local que não favoreceu completamente a remineralização da dentina, diferente do observado no esmalte. Esmalte conseguiu remineralizar nas condições testadas (blocos cobertos com tela para acumular biofilme e exposição à sacarose 3x/dia), entretanto, para a dentina parece ser importante e necessária a remoção mecânica do biofilme, desde que a escovação e fluoreto podem ter efeito aditivo no controle da cárie (Nyvad, 2008). Assim, possivelmente somente o F proveniente dos reservatórios de ("CaF_2") formado pela aplicação de APF no grupo de combinação de meios teve a capacidade de remineralizar significativamente a superfície da dentina-cariada como mostrado na área da lesão de cárie (Cap. 2; Tabela 4; pág. 35). Positivamente para o esmalte, a dureza de superfície demonstrou ser válida para determinar a %RDS a qual correspondeu à área de lesão de cárie (ΔS), validando esta metodologia para o estudo da remineralização em esmalte dentário.

No capítulo 1, além da dureza, a concentração de cálcio no meio de cultura foi avaliada como indicador químico da desmineralização. O gráfico desse valor (Cap. 1; figura 1; pág. 13) representa claramente como o processo foi acontecendo com o tempo, sendo que nos grupos com ausência de F ou menor concentração de F a desmineralização foi maior, sendo concentração de F dependente. No período noturno os valores baixos observados foram congruentes com o esperado

no período *overnight* ao pH neutro, favorecendo a remineralização, sem observar perda mineral que seria refletida pelo aumento de Ca^{++} no meio de cultura.

Como descrito no capítulo 2, novas variáveis de resposta foram acrescentadas, como a análise da concentração de F no fluido do biofilme, a formação de F fracamente e fortemente ligado separadamente, e a dureza em corte. Esta última avaliação foi desconsiderada no modelo *in vitro* devido à menor profundidade das lesões de cárie observada em ensaios prévios (30-40 µm) que dificultaria diferenciar os grupos de tratamento. Além disso, no *in situ* foram avaliados simultaneamente o efeito do F na redução da desmineralização e o efeito na ativação da remineralização. Foi detalhadamente descrito que além de blocos hígidos, a metade deles foi submetida previamente à formação de lesão de cárie no laboratório para ter amostras com lesão de cárie subsuperficial, entretanto, no estudo *in vitro* foram estudados somente esmalte e dentina radicular hígidos.

Os nossos resultados do capítulo 2 foram obtidos simulando condições naturais para o desenvolvimento de cárie de maneira controlada com uso de modelo *in situ*. Nós realizamos diferenças no desafio cariogênico para permitir visualizar o papel do F no desenvolvimento da cárie nos processos de des- e re-. O desafio cariogênico realizado sobre os blocos cariados foi menor (3x/dia) que aquele realizado sobre os hígidos (8x/dia), para evidenciar o efeito do F na remineralização (Re > Des). O desafio cariogênico utilizando sacarose 8x/dia tem sido utilizado com sucesso em estudos *in situ* para desmineralizar esmalte (Paes Leme et al., 2004; Ccahuana-Vasquez et al., 2007) e dentina radicular (Botelho et al., 2014) num curto período de tempo (14 dias) e observar o efeito dos tratamentos mantendo a superfície dentária dos blocos.

Ambos estudos *in situ* prévios (Paes Leme et al., 2004 e Vale et al., 2011) utilizaram delineamento fatorial 2x2, em dois níveis quanto ao dentifrício (fluoretado (1100 ppm F/NaF) ou não fluoretado) e F-gel em dois níveis (aplicação ou não aplicação). Em ambos, a frequência de escovação foi 3x/dia e utilizaram substratos hígidos. O delineamento do trabalho *in situ* (capítulo 2) considerou o uso de dentifrício fluoretado 2x/dia, atentando para o fato de que essa frequência mostra um melhor efeito que 1x/dia (Marinho et al., 2003; Twetman, 2009) e que pelo menos duas

vezes por dia é a indicação clínica mais recomendada (Ellwood et al., 2008; Parnell e O'Mullane, 2013). Paes Leme et al (2004) utilizaram fases de 14 dias cada para avaliar o efeito na redução da desmineralização do esmalte e Vale et al (2011), 7 dias cada fase para o mesmo estudo porém em dentina radicular hígida. Em nosso estudo *in situ*, nós escolhemos trabalhar com o grupo de combinação flúor fosfato acidulado em gel e DF-1100 ppm F, pois há forte evidencia do efeito anticárie do F-gel acidulado (Marinho et al., 2002), além do que termos mostrado previamente efeito sinérgico dessa combinação na redução da desmineralização de dentina (Vale et al., 2011). Também nós utilizamos fases de 14 dias para ambos os substratos com base nos resultados para dentina de Botelho et al. (2014), o qual mostrou que fases de 10 e 14 dias não foram diferentes e legitimou a dureza de superfície na dentina como técnica para detectar diferenças quando usado ou não DF-1100 ppm.

Em relação ao período de *wash-out* entre as fases experimentais do estudo *in situ*, nós tivemos no mínimo três dias, tempo que demostrou ser suficiente para eliminar o F residual na saliva proveniente de dentifrício de alta concentração de F (Fernández et al., 2015). No caso da APF, embora os blocos do nosso experimento apresentem aproximadamente 20% do valor formado inicialmente na APF após 14 dias da aplicação, os reservatórios tipo "CaF_2" formados na superfície dos dentes dos sujeitos de pesquisa não podem alterar a concentração de F na saliva para ter alguma interferência em nosso experimento. Assim, o efeito cruzado do F foi totalmente descartado no nosso estudo *in situ*.

CONCLUSÃO

Os resultados *in vitro* e *in situ* dessa tese permitiram concluir que:

- Um modelo de biofilme foi validado para estimar o efeito dose-resposta de dentifrício fluoretado na redução da desmineralização do esmalte e dentina radicular.

- O efeito de dentifrício fluoretado no controle de cárie de esmalte ou dentina é concentração-dependente, sendo a combinação com aplicação profissional de fluoreto mais relevante para o controle de cárie radicular que coronária.

REFERÊNCIAS*

Bagramian RA, Garcia-Godoy F, Volpe AR. The global increase in dental caries. A pending public health crisis. Am J Dent. 2009;22:3-8.

Baysan A, Lynch E, Ellwood R, Davies R, Petersson L, Borsboom P. Reversal of primary root caries using dentifrices containing 5,000 and 1,100 ppm fluoride. Caries Res. 2001;35:41-6.

Berry TG, Summitt JB, Sift EJ, Jr. Root caries. Oper Dent. 2004;29:601-7.

Botelho JN, Del Bel Cury AA, Silva WJ, Tenuta LM, Cury JA. The effect of fluoride toothpaste on root dentine demineralization progression: A pilot study. Braz Oral Res. 2014;28:1-5.

Bratthall D, Hansel-Petersson G, Sundberg H. Reasons for the caries decline: What do the experts believe? Eur J Oral Sci. 1996;104:416-22; discussion 23-5, 30-2.

Ccahuana-Vasquez RA, Tabchoury CP, Tenuta LM, Del Bel Cury AA, Vale GC, Cury JA. Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride. Caries Res. 2007;41:9-15.

Cury J, Tenuta L, Tabchoury C: Funções e propriedades da saliva; in: Livro do congresso do centenário da apcd. São Paulo, Napoleão, 2011.

Cury JA, Tenuta LM. How to maintain a cariostatic fluoride concentration in the oral environment. Adv Dent Res. 2008;20:13-6.

Cury JA, Tenuta LM. Enamel remineralization: Controlling the caries disease or treating early caries lesions? Braz Oral Res. 2009;23 Suppl 1:23-30.

* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

Cury JA, Tenuta LM. Evidence-based recommendation on toothpaste use. Braz Oral Res. 2014;28 Spec no. 1:1-7.

Cury JA, Tenuta LM, Ribeiro CC, Paes Leme AF. The importance of fluoride dentifrices to the current dental caries prevalence in brazil. Braz Dent J. 2004;15:167-74.

Curzon ME, Preston AJ. Risk groups: Nursing bottle caries/caries in the elderly. Caries Res. 2004;38 Suppl 1:24-33.

Du M, Jiang H, Tai B, Zhou Y, Wu B, Bian Z. Root caries patterns and risk factors of middle-aged and elderly people in china. Community Dent Oral Epidemiol. 2009;37:260-6.

Ekstrand KR, Poulsen JE, Hede B, Twetman S, Qvist V, Ellwood RP. A randomized clinical trial of the anti-caries efficacy of 5,000 compared to 1,450 ppm fluoridated toothpaste on root caries lesions in elderly disabled nursing home residents. Caries Res. 2013;47:391-8.

Ellwood R, Fejerskov O, Cury J, Clarkson B: Fluoride in caries control; in Fejerskov O, Kidd E (eds): Dental caries: The disease and its clinical management. Oxford, Blackwell Munksgaard, 2008, vol 2nd ed, pp 287-323.

Featherstone JD. Fluoride, remineralization and root caries. Am J Dent. 1994;7:271-4.

Featherstone JD, ten Cate JM, Shariati M, Arends J. Comparison of artificial caries-like lesions by quantitative microradiography and microhardness profiles. Caries Res. 1983;17:385-91.

Fernández CE, Tenuta LMA, Cury JA. Wash-out period for crossover design experiments using high fluoride concentration dentifrice Rev Clin Periodoncia Implantol Rehabil Oral. 2015.

Fure S, Gahnberg L, Birkhed D. A comparison of four home-care fluoride programs on the caries incidence in the elderly. Gerodontology. 1998;15:51-60.

Giacaman RA, Munoz MJ, Ccahuana-Vasquez RA, Munoz-Sandoval C, Cury JA. Effect of fluoridated milk on enamel and root dentin demineralization evaluated by a biofilm caries model. Caries Res. 2012;46:460-6.

Griffin SO, Regnier E, Griffin PM, Huntley V. Effectiveness of fluoride in preventing caries in adults. J Dent Res. 2007;86:410-5.

Heijnsbroek M, Paraskevas S, Van der Weijden GA. Fluoride interventions for root caries: A review. Oral Health Prev Dent. 2007;5:145-52.

Herkstroter FM, Witjes M, Arends J. Demineralization of human dentine compared with enamel in a ph-cycling apparatus with a constant composition during de- and remineralization periods. Caries Res. 1991;25:317-22.

Hoppenbrouwers PM, Driessens FC, Borggreven JM. The vulnerability of unexposed human dental roots to demineralization. J Dent Res. 1986;65:955-8.

Jang AT, Lin JD, Choi RM, Choi EM, Seto ML, Ryder MI, et al. Adaptive properties of human cementum and cementum dentin junction with age. Journal of the mechanical behavior of biomedical materials. 2014;39:184-96.

Jones JA. Root caries: Prevention and chemotherapy. Am J Dent. 1995;8:352-7.

Kawasaki K, Featherstone JD. Effects of collagenase on root demineralization. J Dent Res. 1997;76:588-95.

Kidd E, Fejerskov O. Changing concepts in cariology: Forty years on. Dental update. 2013;40:277-8, 80-2, 85-6.

Laheij AM, van Strijp AJ, van Loveren C. In situ remineralisation of enamel and dentin after the use of an amine fluoride mouthrinse in addition to twice daily brushings with amine fluoride toothpaste. Caries Res. 2010;44:260-6.

Marinho VC, Higgins JP, Logan S, Sheiham A. Fluoride gels for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2002:CD002280.

Marinho VC, Higgins JP, Sheiham A, Logan S. Fluoride toothpastes for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2003:CD002278.

Marinho VC, Higgins JP, Sheiham A, Logan S. Combinations of topical fluoride (toothpastes, mouthrinses, gels, varnishes) versus single topical fluoride for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2004:CD002781.

McGrath C, Zhang W, Lo EC. A review of the effectiveness of oral health promotion activities among elderly people. Gerodontology. 2009;26:85-96.

Mukai Y, Lagerweij MD, ten Cate JM. Effect of a solution with high fluoride concentration on remineralization of shallow and deep root surface caries in vitro. Caries Res. 2001;35:317-24.

Munoz-Sandoval C, Munoz-Cifuentes MJ, Giacaman RA, Ccahuana-Vasquez RA, Cury JA. Effect of bovine milk on streptococcus mutans biofilm cariogenic properties and enamel and dentin demineralization. Pediatr Dent. 2012;34:e197-201.

Nordstrom A, Birkhed D. Preventive effect of high-fluoride dentifrice (5,000 ppm) in caries-active adolescents: A 2-year clinical trial. Caries Res. 2010;44:323-31.

Nyvad B: The role of oral hygiene in Fejerskov O, Kidd E (eds): Dental caries the disease and its clinical management. Oxford, Blackwell Munksgaard, 2008, pp 257-64.

Nyvad B, Fejerskov O. Root surface caries: Clinical, histopathological and microbiological features and clinical implications. Int Dent J. 1982;32:311-26.

Nyvad B, Fejerskov O. Active root surface caries converted into inactive caries as a response to oral hygiene. Scand J Dent Res. 1986;94:281-4.

Nyvad B, ten Cate JM, Fejerskov O. Arrest of root surface caries in situ. J Dent Res. 1997;76:1845-53.

Ogaard B, Rolla G, Arends J. In vivo progress of enamel and root surface lesions under plaque as a function of time. Caries Res. 1988;22:302-5.

Ogard B, Seppä L, Rølla G. Professional topical fluoride applications--clinical efficacy and mechanism of action. Adv Dent Res. 1994;8:190-201.

Paes Leme AF, Dalcico R, Tabchoury CP, Del Bel Cury AA, Rosalen PL, Cury JA. In situ effect of frequent sucrose exposure on enamel demineralization and on plaque composition after apf application and f dentifrice use. J Dent Res. 2004;83:71-5.

Parnell C, O'Mullane D. After-brush rinsing protocols, frequency of toothpaste use: Fluoride and other active ingredients. Monogr Oral Sci. 2013;23:140-53.

Petersen PE, Yamamoto T. Improving the oral health of older people: The approach of the who global oral health programme. Community Dent Oral Epidemiol. 2005;33:81-92.

Petersson LG. The role of fluoride in the preventive management of dentin hypersensitivity and root caries. Clin Oral Investig. 2013;17 Suppl 1:S63-71.

Rodrigues JA, Lussi A, Seemann R, Neuhaus KW. Prevention of crown and root caries in adults. Periodontol 2000. 2011;55:231-49.

Sonesson M, Twetman S, Bondemark L. Effectiveness of high-fluoride toothpaste on enamel demineralization during orthodontic treatment--a multicenter randomized controlled trial. Eur J Orthod. 2013.

Srinivasan M, Schimmel M, Riesen M, Ilgner A, Wicht MJ, Warncke M, et al. High-fluoride toothpaste: A multicenter randomized controlled trial in adults. Community Dent Oral Epidemiol. 2014;42:333-40.

Stamm JW, Banting DW, Imrey PB. Adult root caries survey of two similar communities with contrasting natural water fluoride levels. J Am Dent Assoc. 1990;120:143-9.

Tan HP, Lo EC, Dyson JE, Luo Y, Corbet EF. A randomized trial on root caries prevention in elders. J Dent Res. 2010;89:1086-90.

ten Cate JM. Current concepts on the theories of the mechanism of action of fluoride. Acta Odontol Scand. 1999;57:325-9.

ten Cate JM, Buijs MJ, Damen JJ. Ph-cycling of enamel and dentin lesions in the presence of low concentrations of fluoride. Eur J Oral Sci. 1995;103:362-7.

ten Cate JM, Damen JJ, Buijs MJ. Inhibition of dentin demineralization by fluoride in vitro. Caries Res. 1998;32:141-7.

ten Cate JM, Featherstone JD. Mechanistic aspects of the interactions between fluoride and dental enamel. Crit Rev Oral Biol Med. 1991;2:283-96.

ten Cate JM, Larsen MJ, Pearce EIF, Fejerskov O: Chemical interactions between the tooth and oral fluids. In: ; in Fejerskov O KE (ed): Dental caries the disease and its clinical management. Oxford, Blackwell Munksgaard, 2008, vol 2ed, pp 210-30.

Tenuta L, Cury J: Fluoreto: Da ciência à práctica clínica; in: Odontologia para crianças e adolescentes. Sao Paulo, 2004.

Tenuta LM, Cerezetti RV, Del Bel Cury AA, Tabchoury CP, Cury JA. Fluoride release from caf2 and enamel demineralization. J Dent Res. 2008;87:1032-6.

Tenuta LM, Cury JA. Fluoride: Its role in dentistry. Braz Oral Res. 2010;24 Suppl 1:9-17.

Tenuta LM, Cury JA. Laboratory and human studies to estimate anticaries efficacy of fluoride toothpastes. Monogr Oral Sci. 2013;23:108-24.

Tenuta LM, Zamataro CB, Del Bel Cury AA, Tabchoury CP, Cury JA. Mechanism of fluoride dentifrice effect on enamel demineralization. Caries Res. 2009;43:278-85.

Twetman S. Caries prevention with fluoride toothpaste in children: An update. Eur Arch Paediatr Dent. 2009;10:162-7.

Vale GC, Tabchoury CP, Del Bel Cury AA, Tenuta LM, ten Cate JM, Cury JA. Apf and dentifrice effect on root dentin demineralization and biofilm. J Dent Res. 2011;90:77-81.

Walls AW, Meurman JH. Approaches to caries prevention and therapy in the elderly. Adv Dent Res. 2012;24:36-40.

Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X. Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2010:CD007868.

Wefel JS. Root caries histopathology and chemistry. Am J Dent. 1994;7:261-5.

White DJ. Reactivity of fluoride dentifrices with artificial caries. I. Effects on early lesions: F uptake, surface hardening and remineralization. Caries Res. 1987;21:126-40.

Wierichs RJ, Meyer-Lueckel H. Systematic review on noninvasive treatment of root caries lesions. J Dent Res. 2014.

Zero DT. In situ caries models. Adv Dent Res. 1995;9:214-30; discussion 31-4.

Zimmer S, Bizhang M, Seemann R, Witzke S, Roulet JF. The effect of a preventive program, including the application of low-concentration fluoride varnish, on caries control in high-risk children. Clin Oral Investig. 2001;5:40-4.

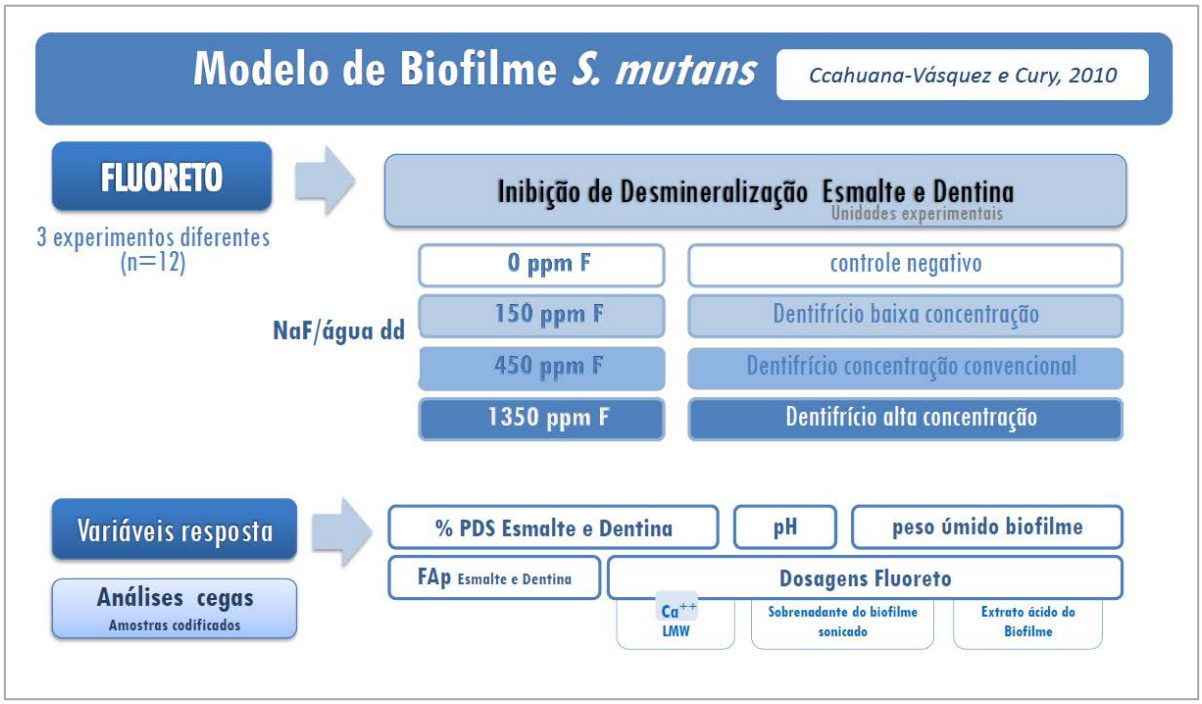
APÊNDICE 1. *Artigos publicados durante o período do Doutorado*

- Tenuta LMA., **Fernández CE**., Brandao AC, Cury J.A. Titratable acidity of beverages influences salivary pH recovery. *Braz Oral Res*. **2015**;29(1);1-6. DOI: 10.1590/1807-3107 BOR-2015.vol29.0032
- **Fernández CE.,** Tenuta LMA., Zárate P, Cury JA. Insoluble NaF in Duraphat® may prolong fluoride reactivity of varnish retained on dental surfaces. *Braz Dent J*. **2014** Apr;25(2):160-4.
- **Fernández CE**., Giacaman RA., Cury JA. Fluoride concentration in bottled waters sold in Chile: importance in caries and dental fluorose. *Rev Med Chile* **2014**; 142: 623-629.
- Giacaman RA, Carrera CA, Muñoz-Sandoval C**, Fernández C**, Cury JA. Fluoride content in toothpastes commercialized for children in Chile and discussion on professional recommendations of use. *Int J Paediatr Dent.* **2013** Mar;23(2):77-83.
- Giacaman RA., **Fernández CE**., Díaz N. Fermentable carbohydrate dietary consumption measured by a cariogenicity scoring system and caries experience in youth and adults. *Rev. Chil. Nutr.* **2012.** 39(4): 116-122.
- **Fernández** González C, Núñez Franz L, Diaz Sanzana N. Oral health determinant in 12 year-old population. *Rev Clin Periodoncia Implantol Rehabil Oral*, **2011**; 4(3): 117-121.

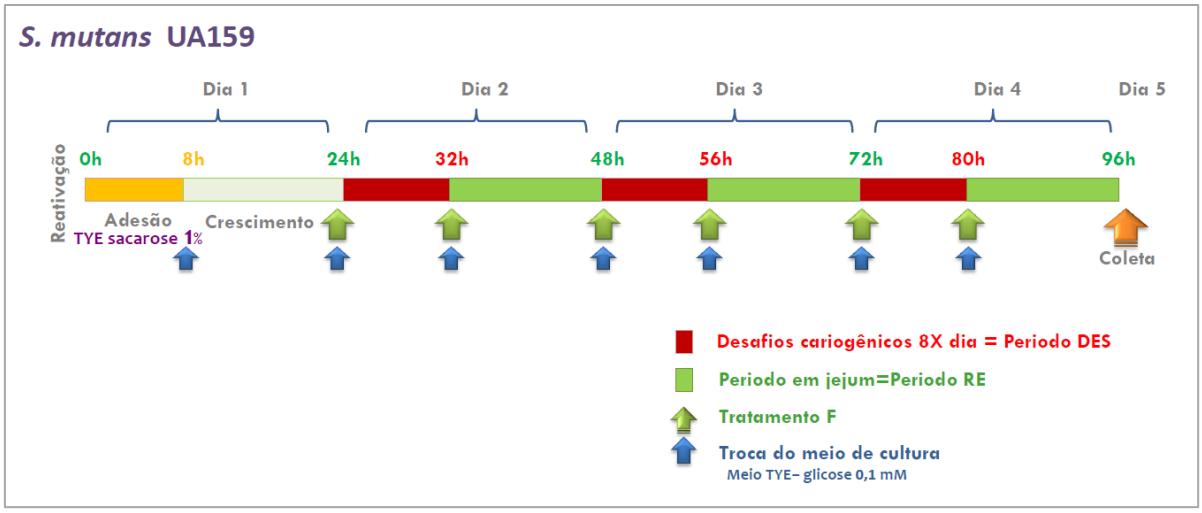
Aceitos

- **Fernández CE**., Tenuta LMA., Cury JA. (**2015**). Wash-out period for crossover design experiments using high fluoride concentration dentifrice. *Rev Clin Periodoncia Implantol Rehabil Oral*. ref. PIRO-D-14-00009.
- **Fernández CE**., González-Cabezas, C. (**2015**). Update on Fluoride Products used for Prevention and Management of Dental Caries. *Dimensions of Dental Hygiene.*

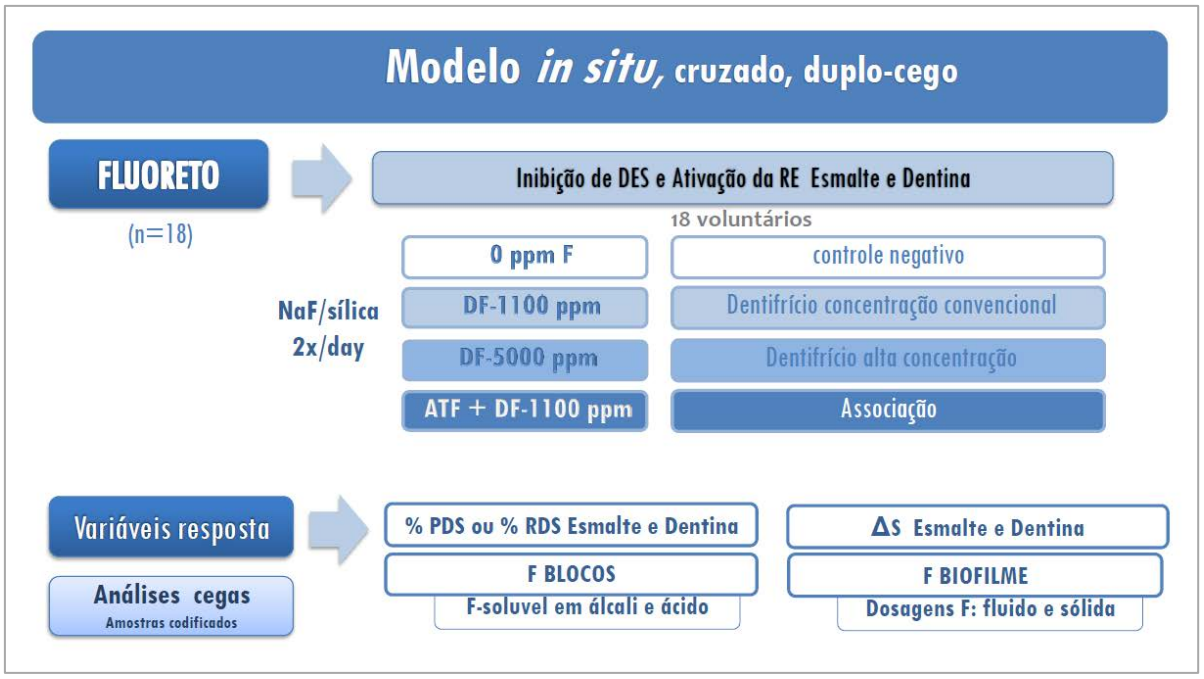
APÊNDICE 2. *Resumo do desenho experimental do capítulo 1 (slide da aula)*



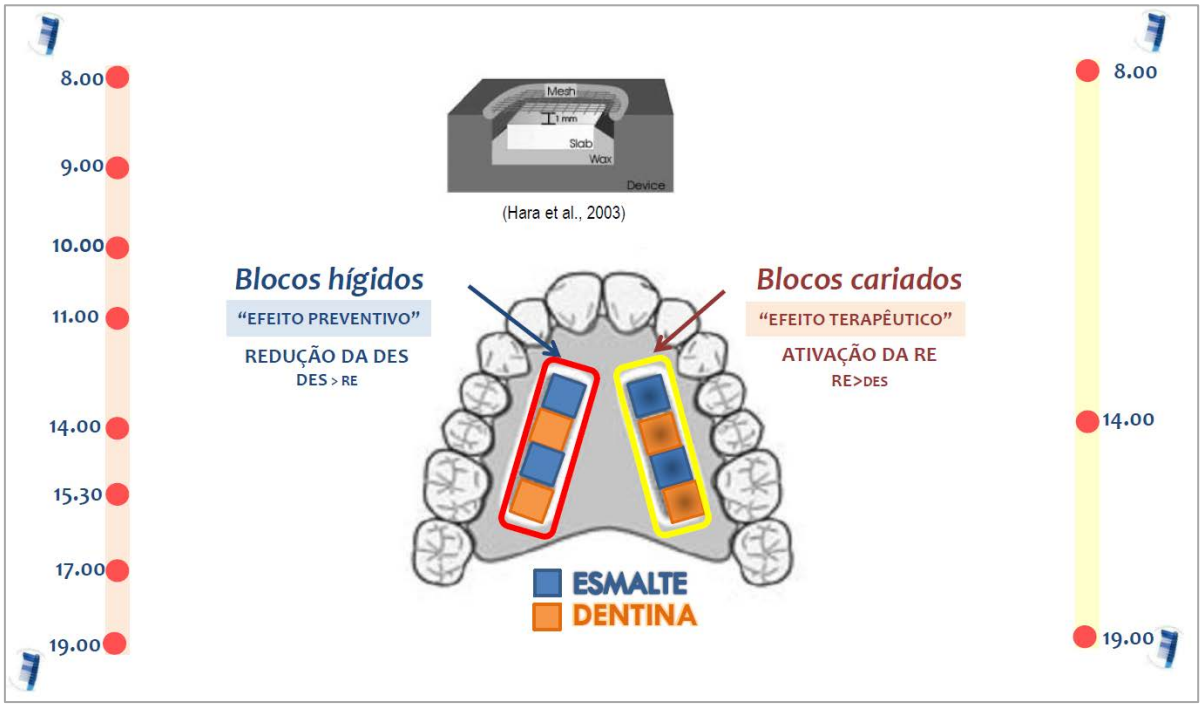
APÊNDICE 3. *Fluxograma do experimento do capítulo 1 (slide da aula).*



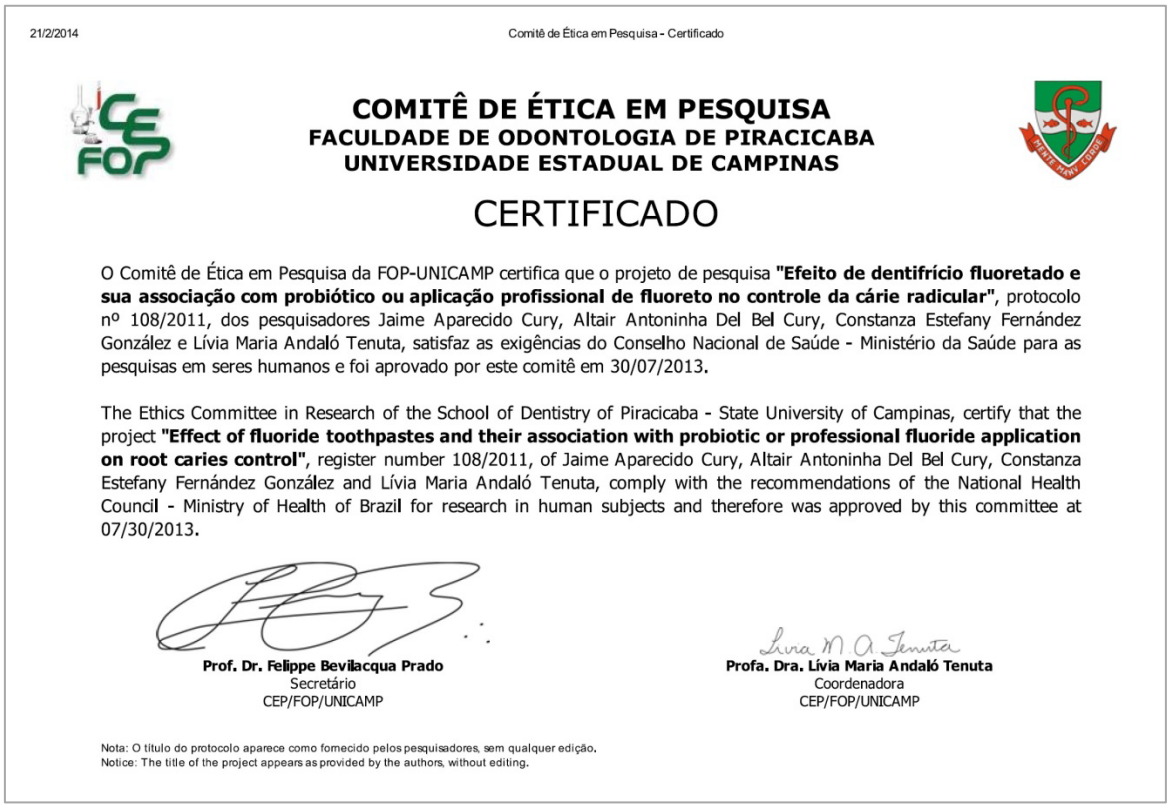
APÊNDICE 4. *Resumo do desenho experimental do capítulo 2 (slide da aula)*



APÊNDICE 5. *Posição dos blocos no aparelho palatino do capítulo 2 (slide da aula).*



ANEXO 1. *Certificado do Comitê de Ética em Pesquisa FOP-UNICAMP.*



21/2/2014 Comitê de Ética em Pesquisa - Certificado

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Efeito de dentifrício fluoretado e sua associação com probiótico ou aplicação profissional de fluoreto no controle da cárie radicular"**, protocolo nº 108/2011, dos pesquisadores Jaime Aparecido Cury, Altair Antoninha Del Bel Cury, Constanza Estefany Fernández González e Lívia Maria Andaló Tenuta, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 30/07/2013.

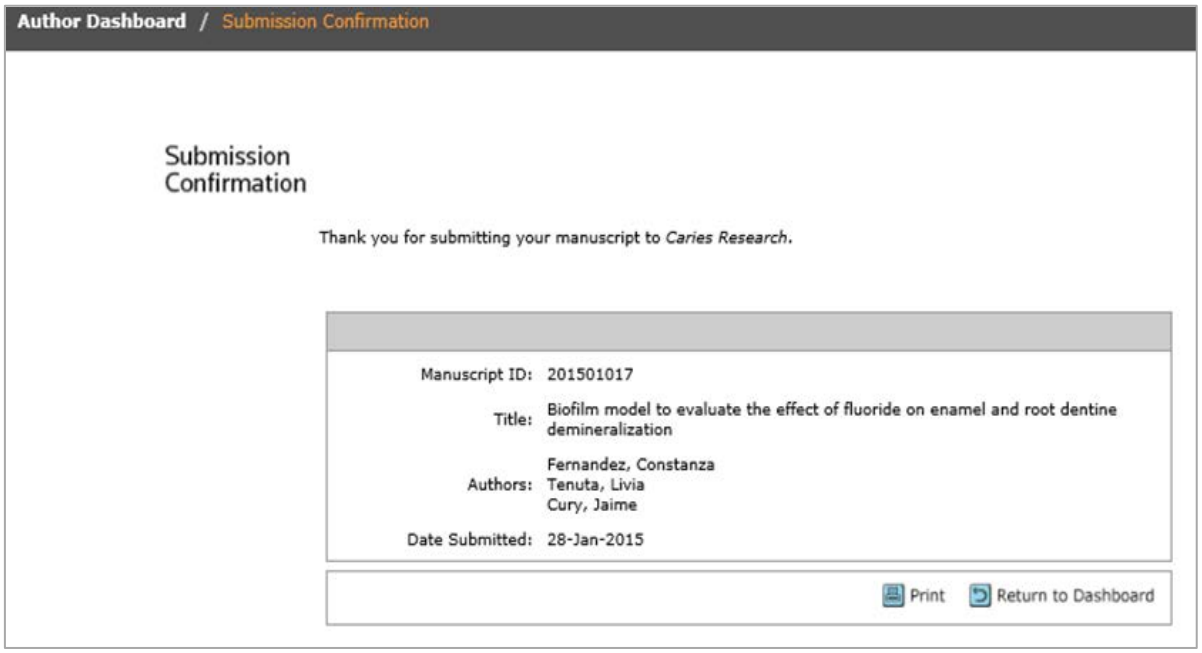
The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Effect of fluoride toothpastes and their association with probiotic or professional fluoride application on root caries control"**, register number 108/2011, of Jaime Aparecido Cury, Altair Antoninha Del Bel Cury, Constanza Estefany Fernández González and Lívia Maria Andaló Tenuta, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 07/30/2013.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2. *Comprovante da submissão do artigo do Capítulo 1*



ANEXO 3. *Declaração*

DECLARAÇÃO

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Tese de Doutorado intitulada "EFEITO DE DENTIFRÍCIO FLUORETADO E APLICAÇÃO PROFISSIONAL DE FLUORETO NO CONTROLE DE CÁRIE DE ESMALTE E DE DENTINA RADICULAR", não infringem os dispositivos da Lei nº 9.610/98, nem o direito autoral de qualquer editora.

Piracicaba, 30 de Dezembro de 2014.

CONSTANZA ESTEFANY FERNANDEZ GONZALEZ
RG: 16001754-6
Autor(a)

JAIME APARECIDO CURY
RG: 4.329.838
Orientador(a)